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THE UNIVERSITY OF ALBERTA

CONCENTRATION AND TOXICITY OF MICROCYSTIN-LR
IN COMPARTMENTS AND ORGANISMS OF AQUATIC FOOD WEBS

BY

BRIAN GLENN KOTAK



A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOLOGICAL SCIENCES

EDMONTON, ALBERTA

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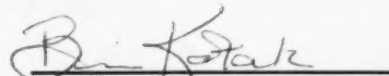
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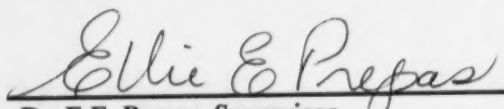
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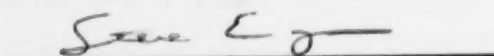
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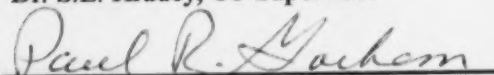
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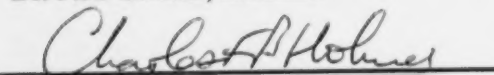
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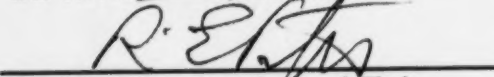
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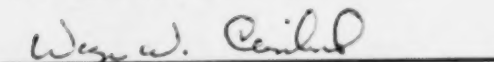

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This thesis is dedicated to two very special people. Firstly, to the memory of Walter Pacholek, my Uncle, who instilled within me the desire to understand and appreciate what lies beneath the waters' surface. Secondly, to Prof. Gordon Robinson, teacher, listener, friend. Your enthusiasm towards teaching and research opened my mind to the exciting world of LRGTs (little round green things).

Abstract

Microcystin-LR (MC-LR) is one of the most commonly-occurring of more than 50 toxic peptides produced by cyanobacteria in eutrophic lakes worldwide. Accidental ingestion of microcystins has caused poisonings and deaths in animals and has been related to gastrointestinal illness and liver cancer in humans. In this thesis, I studied the concentration of MC-LR in water and organisms of lakes, relationship of MC-LR concentrations to environmental variables, and the toxicity to fish (rainbow trout), in comparison to mammals (mice).

Over the five years of study, MC-LR (detected by high performance liquid chromatography) was found in 24 of 28 Alberta lakes, 6 of 11 farm dugouts and one of three storm water retention ponds located in the City of Edmonton.. Concentrations ranged from just above the detection limit (approx. $1 \mu\text{g g}^{-1}$ dry wieght plankton biomass) to over $1500 \mu\text{g g}^{-1}$, and as high as $6400 \mu\text{g L}^{-1}$ of lake water when expressed on a volumetric basis.

MC-LR concentration in the phytoplankton was most variable within and between years in lakes, and to a lesser extent, spatially within a lake. This variability was associated with changes in the abundance and biomass of the cyanobacterium *Microcystis aeruginosa* (r up to 0.77). Additionally, MC-LR concentration in *M. aeruginosa* decreased more than 6-fold at night relative to daytime concentrations. MC-LR concentration in phytoplankton was likely regulated by environmental factors that affected the biomass of *M. aeruginosa* and the concentration of toxin in the cells. Both *M.*

aeruginosa biomass and MC-LR in *M. aeruginosa* cells were strongly correlated with total phosphorus concentration, but only *M. aeruginosa* biomass was related to the ratio of total nitrogen to total phosphorus (TN:TP), and inorganic nitrogen ($\text{NO}_2^- + \text{NO}_3^-$, NH_4^+) concentration. MC-LR concentration in phytoplankton therefore, may be regulated primarily by the nutrients phosphorus and nitrogen, suggesting that nutrient (particularly phosphorus) loading to lakes may increase the incidence and concentration of cyanobacterial toxins in water bodies. This is the first study to relate concentrations of any cyanobacterial toxin to limnologically-important variables.

MC-LR concentrations in water ($< 0.45 \mu\text{m}$) and phytoplankton ($>64 \mu\text{m}$) collected from four lakes increased with lake trophic status. Maximum MC-LR concentrations ranged from non-detectable in water and phytoplankton in an oligo-mesotrophic lake, to 0.34 and $11.2 \mu\text{g L}^{-1}$, respectively, in a hypereutrophic lake. Aqueous microcystin concentration, determined by protein phosphatase bioassay, generally paralleled MC-LR concentration in phytoplankton. MC-LR was also detected in zooplankton communities ($>243 \mu\text{m}$, at concentrations up to $67 \mu\text{g g}^{-1}$ of dry weight biomass) and MC-LR concentrations in zooplankton and phytoplankton were correlated ($r=0.69$). Of nine groups of macroinvertebrates analyzed, MC-LR, at concentrations up to $120 \mu\text{g g}^{-1}$, was detected only in gastropods. This is one of few studies to demonstrate that cyanobacterial toxins can be transferred to aquatic invertebrates.

In laboratory toxicity trials, an intraperitoneal (i.p.) dose of $\geq 50 \mu\text{g kg}^{-1}$ MC-LR killed 90-100% of the mice, while i.p. doses as high as $800 \mu\text{g kg}^{-1}$ caused only 60%, and

oral doses as high as $8,000 \mu\text{g kg}^{-1}$ only 20% mortality in rainbow trout. Further, mean survival time in mice given a lethal dose of MC-LR was up to 30 times shorter than in fish.

Lesions were evident in both the liver and kidney in fish. The main lesion observed in the livers of fish was liquifactive necrosis (rupture of the plasma membrane leaving organelles floating in a milieu of cellular debris). Plasma activities of lactate dehydrogenase, and aspartic acid and alanine aminotransferases were significantly higher in MC-LR-exposed fish than control fish in i.p. and oral exposure trials, indicative of hepatic injury. Lower plasma enzyme activities and a reduced extent and severity of liver damage in fish from the oral relative to the i.p. trial suggested a low efficiency of transport of the toxin from the digestive tract. Kidney lesions in the fish at the highest doses consisted of dilation of Bowman's space and necrosis of tubular epithelium of the kidney. Additionally, necrosis of the villi of pyloric caecae and the intestine was observed in fish in the oral trial.

My studies indicate that MC-LR is much more prevalent in eutrophic lakes than is suggested by the limited number of published studies, and that the concentration of toxin may be regulated by the concentrations of the nutrients phosphorus and nitrogen. Concentrations of MC-LR detected in aquatic invertebrates, accumulated by grazing on phytoplankton from the hypereutrophic lakes, may be sufficient to cause adverse health effects including damage to the liver, kidney and gastrointestinal tract in fish.

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Chapter 1

General Introduction and Scope of Thesis

Microcystin-LR (MC-LR) is one of more than 50 cyclic peptide hepato-(liver)-toxins that are produced predominantly by the cyanobacterium (blue-green alga), *Microcystis aeruginosa*, in fresh waters (Carmichael 1994; Rinehart et al. 1994). It is also one of the most toxic (intraperitoneal LD₅₀ of 50 µg kg⁻¹ in mice, Carmichael 1988) and is likely the most widely occurring microcystin analogue worldwide. Each year, accidental consumption of phytoplankton or water containing cyanobacterial toxins has been related to poisonings and deaths of livestock and wildlife, at times in spectacularly large numbers (e.g., >1,000 animals; Pybus et al. 1986). Chronic exposure to microcystins in drinking water is also suspected to be related to high rates of primary liver cancer in humans in some regions of the world (Carmichael 1994).

Because animal poisoning incidents occur sporadically and are not publicized to any extent, the occurrence of toxic forms of cyanobacteria in fresh water has traditionally been assumed to be limited. In the late 1980s and early 1990s, systematic studies documenting the occurrence of toxic blooms in lakes, with detection of the toxins by mouse bioassays, indicated that the occurrence of toxic cyanobacteria in lakes was much more widespread than surmised based on animal poisonings alone (Codd et al. 1989; Lanaras et al. 1989; Repavich et al. 1990; Sivonen et al. 1989). Very few studies have attempted to assess the extent and concentration of cyanobacterial toxins in freshwater lakes by more sensitive methods of detection (such as by high performance liquid chromatography [HPLC]) and most of these are limited to only one or two lakes, and of short (< 1 yr) duration (Lindholm and Meriluoto 1991; Watanabe et al. 1989; Shirai et al. 1991). Only one study has assessed microcystin concentrations in phytoplankton for > 1 yr, but was limited to only one water body (Hartbeespoort Dam, South Africa, Wicks and Thiel 1990). Therefore, an assessment of the distribution and concentration of cyanobacterial toxins in any region of the world by sensitive methods of detection is lacking. Such an assessment is needed in western Canada, given the high proportion of eutrophic lakes.

The factors (environmental, genetic) that may control the production of cyanobacterial toxins in phytoplankton species are poorly understood. Field data that relate cyanobacterial toxin concentrations in lakes to limnological or environmental parameters are lacking. Therefore, management of toxic blooms in lakes is always done on an after-the-fact basis (usually by eliminating the bloom by use of a herbicide). Laboratory studies which have examined the effects of manipulating culture media conditions (e.g., nutrient concentrations, pH) or environmental variables (e.g., light, temperature) have provided conflicting results (Watanabe and Oishi 1985; Sivonen 1990; Rapala et al. 1993). Since these experiments utilize laboratory strains of species which may have been in culture for up to a decade or more, and because usually one factor is manipulated at a time (e.g., phosphorus concentration) while other factors (e.g., light, temperature) are held at saturating conditions, their applicability to natural aquatic environments is questionable. While the occurrence of toxic phytoplankton in marine systems has been assessed, the large spatial scale of such systems can severely limit the ability to relate toxin concentrations to environmental variables. In contrast, lakes provide an excellent opportunity to examine such relationships due to their small size. Only one field study has attempted to correlate microcystin concentrations in phytoplankton communities in a natural lake setting (Wicks and Thiel 1990) to identify which environmental factors may influence toxin production. However, the study was limited to one water body, and because of its location (South Africa) extrapolation of their results to North America (particularly where ice-cover is possible for > 6 mo of the year) is not likely valid. Therefore, no studies have yet attempted to determine the occurrence and concentration of any cyanobacterial toxin on a regional (Provincial, State) basis and relate *in situ* toxin concentrations to limnological and/or environmental parameters. Until this is done, the safe management of toxic blooms will remain reactive rather than proactive.

Given the close association between toxin-producing cyanobacteria and aquatic organisms such as zooplankton, gastropods and fish, one might expect a certain degree of accumulation of MC-LR in those organisms that feed directly on the cyanobacteria. This has rarely received attention. Watanabe et al. (1992) detected high concentrations (up to 1387 $\mu\text{g g}^{-1}$ of tissue) of three microcystin analogues in a zooplankton community

dominated by *Bosmina fatalis* in Lake Kasumiguara, Japan. This study however was limited to one lake with only a few samples collected, was restricted to zooplankton and did not attempt to elucidate factors which may influence the accumulation of the toxins in the zooplankton. Therefore, virtually nothing is known about the accumulation of MC-LR in organisms of different trophic levels in aquatic food webs and how accumulation may be related to lake trophic status (e.g., oligo-, meso-, eutrophic).

The pathological effects of different microcystin analogues have been studied in detail in several mammalian species (livestock such as cattle, sheep, pigs, chickens, and in laboratory mice and rats). Little attention has been directed to determining if microcystins can have a negative impact on aquatic organisms such as fish. Oral or intraperitoneal exposure to MC-LR can cause massive destruction of the liver in rats and mice and death in less than 2 h (Falconer et al. 1981; Hooser et al. 1989). The primary lesion in the liver is hemorrhage, with the liver usually doubling in mass due to the influx of blood (Falconer et al. 1981; Brooks and Codd 1987). In the absence of adequate dissolved oxygen or ammonia field data, cyanobacterial toxins are often assumed to cause or contribute to massive fish kills in lakes during the summer months on the prairies, always without any scientific evidence. Only two studies have examined the uptake and toxicity of MC-LR in fish by a natural route of exposure (oral exposure, Sugaya et al. 1990; Tencalla et al. 1994) and none have examined uptake across the gill surface. Only one study has assessed the pathological effects of MC-LR in fish in detail (Raberg et al. 1991) and although highly toxic to the fish (common carp), pathological changes in the liver differed from those observed in mammals and damage was not restricted to only the liver (e.g., kidney damage was also reported), as is the case in mammals. A systematic comparison of the toxicity and pathology of MC-LR in mammals and fish should be conducted to fully investigate these differences.

This thesis examined aspects of the occurrence, accumulation and toxicity of MC-LR from Alberta water bodies, including: 1) occurrence, factors potentially affecting, and correlation of MC-LR concentration in phytoplankton in eutrophic and hypereutrophic lakes (Chapters 3, 4 and 5), occurrence and concentration of MC-LR in various components (water, phytoplankton, aquatic invertebrates and fish) of aquatic food webs in

lakes of varying trophic status (Chapter 6) and an assessment of the toxicity and pathological effects of MC-LR in mice and rainbow trout (Chapters 3, 7 and 8).

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Chapter 2

Literature Review - Cyanobacterial Toxins

1. General Introduction

1.1 Cyanobacteria - Cyanobacteria (blue-green algae) often dominate the phytoplankton communities of lakes, reservoirs, small ponds, slow-flowing rivers and even large seas, worldwide during the summer and autumn months. Their widespread occurrence in such systems is often attributed to their physiological adaptations (such as the ability to regulate their buoyancy in the water column) that may give them a competitive advantage over other algal groups in the phytoplankton, and their poor utilization as a food resource by suspension-feeding zooplankton (Reynolds 1987; Paerl 1988). These characteristics may allow cyanobacteria to proliferate in water bodies to such an extent that massive surface accumulations or "blooms" may result. These blooms can greatly impair water quality, creating many undesirable problems. These can include: loss of tourism revenue due to the reduced aesthetic value of a water body, increased frequency and magnitude of summer fish kills caused by depletion of dissolved oxygen concentration in the water column after a cyanobacterial bloom decomposes (Barica 1975), and an increased cost of drinking water treatment to adequately remove particulate matter and dissolved tastes and musty odors produced by the cyanobacteria (Persson 1983; Hayes and Burch 1989).

Cyanobacterial blooms may also directly affect human health. Chlorination of drinking water containing excessive organic material (present as cyanobacteria) during water treatment may result in the production of trihalomethanes (Palmstrom et al. 1988), compounds suspected of being carcinogenic. Additionally, several genera of cyanobacteria produce contact irritants which may cause skin rashes, vomiting and diarrhea after recreational contact with the water.

2. Cyanobacterial Toxins

Concerns of the acute toxicity of cyanobacteria to livestock, wildlife and humans have also focused much attention on the occurrence of "toxic" forms of cyanobacteria in

water bodies. Francis (1878) gave the first published account of mortality of domestic animals (i.e., sheep, horses, pigs and dogs) caused by the accidental consumption of toxic cyanobacteria. Animals died after drinking water along the shores of Lake Alexandrina, Australia, during a bloom comprised mainly of the cyanobacterium, *Nodularia spumigena*. However, accounts of animal poisonings attributed to blooms of toxic cyanobacteria may extend back well before the time of Francis, descriptions which may be written in personal memoirs or diaries. Freshwater cyanobacteria produce two classes of toxins: neurotoxins and hepato-(liver)-toxins.

2.1 Neurotoxins - Neurotoxins are compounds which affect the nervous system, and if ingested in sufficient concentration can cause respiratory arrest and death in five to 30 min (Carmichael et al. 1985). Freshwater cyanobacterial neurotoxins are produced by species and strains of *Anabaena*, *Aphanizomenon*, *Oscillatoria* and *Nostoc* and include four principle toxins: anatoxin-a, anatoxin-a(s) and saxitoxin and neosaxitoxin (the latter two are also known as paralytic shellfish poisons [PSP]; Table 1).

Saxitoxin and neosaxitoxin (Fig. 1) act by blocking sodium channels at nerve synapses and thus interfere with the propagation of impulses along nerves (Adelman et al. 1982). They are also very toxic, with an LD₅₀ (intraperitoneal, in mice) of 10 µg kg⁻¹ (Carmichael et al. 1985; Table 1). PSPs, including saxitoxin and neosaxitoxin, are more commonly associated with the marine environment however. PSPs are produced by the dinoflagellates *Protogonyaulax tamarensis* and *P. catenella*, (Hall et al. 1980; Carmichael et al. 1985) and have been responsible for the periodic closing of shellfish harvesting operations worldwide. With respect to contaminated shellfish, PSPs have caused serious poisonings and even fatalities when humans consumed shellfish that contained lethal concentrations of PSP, toxin accumulated by the shellfish while filter feeding on the toxic dinoflagellates (reviewed by Carmichael et al. 1985). Of the freshwater cyanobacteria studied to date, only *Aphanizomenon flos-aquae* (Ikawa et al. 1982; Mahmood and Carmichael 1986a) and *Anabaena circinalis* (Humpage et al. 1993) have been reported to produce saxitoxin and neosaxitoxin.

Anatoxin-a (Table 1; Fig. 2a) is a secondary amine which is produced by *An. flos-aquae*, *An. spiroides*, *Oscillatoria agardhii*, *O. rubescens*, *Aph. flos-aquae* and *Nostoc*

(Huber 1972; Devlin et al. 1977; Sivonen et al. 1989a; Edwards et al. 1992; Skulberg et al. 1992; Rapala et al. 1993). It is a potent neuromuscular depolarizing agent which causes over-excitation, and finally fatigue of the muscles involved with breathing (Carmichael 1994). The mode of action of anatoxin-a is identical to that of the neurotransmitter, acetylcholine. However, unlike acetylcholine, which is broken down rapidly to ensure that excessive muscle contraction and fatigue do not occur, anatoxin-a is not broken down within the body. Thus, it is an irreversible neuromuscular stimulant. Accidental ingestion of a lethal dose of anatoxin-a results in rapid (in as little as five min) respiratory arrest. Signs of anatoxin-a poisoning in animals include involuntary muscle twitching and contraction, muscle spasms, gasping for breath, convulsions and ultimately, death (Carmichael 1992). Anatoxin-a has an LD₅₀ of 250 µg kg⁻¹ (intraperitoneal in mice; Table 1).

Although similar in name, anatoxin-a(s) is structurally distinct from anatoxin-a. Anatoxin-a(s) is an organophosphate ester (Table 1; Fig. 2b) with similar toxicological characteristics to the synthetic insecticides malathion and parathion. Signs of poisoning include all those mentioned for anatoxin-a, along with profuse salivation (hence the designation -a(s)) (Mahmood and Carmichael 1986b). Anatoxin-a(s) has only been reported to be produced by *An. flos-aquae* (Mahmood and Carmichael 1986b) and *O. agardhii* (Hawser et al. 1990). The mode of action is also distinct from that of anatoxin-a. Under normal conditions in the body, acetylcholine, the neurotransmitter responsible for propagating nerve impulses between axons and between axons and muscles, is broken down by acetylcholinesterases after a nerve impulse has been transmitted. Anatoxin-a(s) inhibits acetylcholinesterase, thus prolonging the depolarizing action of acetylcholine on the muscles involved in breathing (Mahmood and Carmichael 1987; Cook et al. 1989; Hyde and Carmichael 1991). Death therefore, is due to respiratory arrest. The LD₅₀ of anatoxin-a(s) is 20 µg kg⁻¹ in mice (Mahmood and Carmichael 1986b).

2.2 Hepatotoxins - Hepatotoxins are the most frequently reported cause of animal and human poisonings that involve freshwater cyanobacterial toxins. This is probably due to the cosmopolitan distribution of the main producer of this class of toxin: the cyanobacterium, *Microcystis* spp. Two sub-groups of cyanobacterial hepatotoxins have

been identified: microcystins, a structurally-conservative group of more than 50 cyclic heptapeptides (peptides composed of seven amino acids) and a smaller group of cyclic pentapeptides termed nodularins. As this thesis deals almost exclusively with microcystins, a more in-depth discussion of the hepatotoxins, as compared to the neurotoxins, follows.

3. Hepatotoxin Structures, Producers and Toxin Stability

3.1 Microcystins - The microcystins comprise a family of more than fifty closely related monocyclic peptide toxins. The name microcystin is derived from the primary producer of these hepatotoxins, the cyanobacterium *Microcystis* spp., although other genera of cyanobacteria also produce microcystins. The generalized structure of microcystin is cyclo (-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-) (Carmichael et al. 1988a) where D-Ala is alanine (in the D configuration), D-MeAsp is D-erythro- β -methylaspartic acid, D-Glu is glutamic acid, and Mdha is N-methyldehydroalanine. X and Z are two L-amino acids which may vary among the microcystins, giving rise to some of the structural analogues (Table 2). Microcystin-LR (MC-LR), one of the most common microcystins, contains leucine (L) and arginine (R) as its two variable amino acids (Fig. 3). Adda is a very unusual and unique C-20 amino acid that has not been reported in any other class of organisms and has the following structural formula: (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid (Botes et al. 1984; Rinehart et al. 1988). Adda is an important constituent of cyanobacterial hepatotoxins in that it: 1) confers toxicity to the compound (Dahlem 1989; Harada et al. 1990a+b), and 2) is hydrophobic and thus may aid in the transport of microcystins across biological membranes (Botes et al. 1982a).

Variations of the generalized microcystin structure give over 50 compounds with toxicities ranging from 50 to 1200 $\mu\text{g kg}^{-1}$ (intraperitoneal LD_{50} in mice; Table 2). Variation occurs through several means: substitution of amino acid residues at the 1, 2, 4, 6 and 7 positions (Namikoshi et al. 1992b+d; Sivonen et al. 1992a), demethylation of D-MeAsp and N-Mdha (amino acid residues 3 and 7 respectively; Namikoshi et al. 1992b; Luukkainen et al. 1994), esterification of D-Glu (amino acid residue 6; Namikoshi et al.

1992b), and modification of Adda (Table 2). Modification of the Adda residue includes replacement of the methoxyl group at the C-9 position with an acetoxyl group giving 0-acetyl-0-demethylAdda (e.g., [ADMAdda⁵]MC-LR; Namikoshi et al. 1990) and demethylation of Adda at the C-9 position giving 0-demethylAdda (e.g., [DMAdda⁵]MC-LR; Namikoshi et al. 1992b). Additionally, during the purification of microcystins by HPLC from cyanobacterial extracts, a small peak is often associated closely with either MC-LR or -RR (Fig. 4a+b; Harada et al. 1990a). These two small peaks represent geometric isomers of their parent microcystins (-LR and -RR) at the C-7 position of Adda. Both minor components are non-toxic (i.e., LD₅₀ > 1,200 µg kg⁻¹ in mice) and are termed [6(z)Adda⁵]MC-LR and [6(z)Adda⁵]MC-RR (Harada et al. 1990a+b; Table 2). Since serine is a biosynthetic precursor of dehydroalanine (Dha), the microcystin analogues which contain serine (as either L-Ser or Mser) are likely precursors of their corresponding dehydroamino acid derivatives (Namikoshi et al. 1992b,c+d). Most microcystins have an LD₅₀ between 50 and 250 µg kg⁻¹ (intraperitoneal in mice; Table 2). Thus many of the variations in structure caused by amino acid substitution (at amino acid residues 1, 2, 4, 6 and 7) have little effect on the toxicity of the compound. One exception is MC-RR, whose toxicity is up to 12-fold lower than MC-LR (Table 2). Similarly, demethylation also seems to have little effect on toxicity since [D-Asp³] and [Dha⁷]MC-LR are nearly as toxic as their methyl analogues. However, esterification of the Glu amino acid residue (a naturally-occurring microcystin) leads to a non-toxic compound. Modifications to the Adda residue can either lead to loss of toxicity (e.g., isomerization at the C-7 position) or not alter toxicity appreciably (e.g., demethylated Adda analogues). Dihydromicrocystins have been synthesized in the laboratory by sodium borohydride reduction with tritium for radiolabel studies. Reduction of MDha, D-MeAsp or D-Glu amino acids in the microcystin peptides did not alter the toxicity of the compounds relative to the parent toxins (Robinson et al. 1989; Meriluoto et al. 1990b). It is of interest to note that the abilities of microcystins and nodularins to inhibit protein phosphatases and promote tumor formation (discussed in detail later) are identical to that of okadaic acid, a structurally unrelated marine dinoflagellate toxin responsible for diarrhetic shellfish poisoning. Structural modeling by Quinn et al. (1993) indicate that the carboxyl group of okadaic

acid occupies a similar position to that of the carboxyl group of the glutamic acid residue in microcystins and nodularin. Both the methyl ester of okadaic acid and of MC-LR at these positions are non-toxic (Nishiwaki et al. 1990).

Microcystins have been isolated from both mixed bloom samples (i.e., phytoplankton samples containing more than one genera or species) and also from unialgal or axenic single species cultures. They have also been isolated from cultures of strains within a species. Cyanobacteria known to produce microcystins include *Microcystis aeruginosa*, *M. viridis*, *M. wessenbergii*, *M. flos-aquae*, *Anabaena flos-aquae*, *An. circinalis*, *An. spiroides*, *An. lemermannii*, *Nostoc* sp., *Oscillatoria agardhii* var. *isothrix*, and *O. agardhii* var. *rubescens* (Botes et al. 1985; Meriluoto et al. 1989; Watanabe et al. 1989, 1991; Harada et al. 1990a, 1991a+b; Sivonen 1990; Sivonen et al. 1990a, 1992a+b; Namikoshi et al. 1990, 1992b+c; Craig et al. 1993; Luukkainen et al. 1993, 1994). Production of certain microcystin analogues does not appear to be restricted to certain species of *Microcystis* or other genera of cyanobacteria. It is interesting to note that not all strains of a species capable of producing microcystins in fact produce the toxins, a dilemma which still has the scientific community perplexed.

Microcystins are water soluble compounds. This is evident by the choice of solvents that are used for the extraction of microcystins from cyanobacterial cells: water, methanol and/or acetic acid. The solubility constants of the microcystins however, have yet to be reported. Despite their high aqueous solubility, microcystins also contain hydrophobic amino acid residues (particularly Adda) which also give the compounds a slightly hydrophobic nature. This is evident when one considers the type of column packing most commonly used in the HPLC analysis of microcystins: microcystins readily interact with hydrophobic reverse phase C-18 packings

Microcystins are remarkably stable in water, although several mechanisms for the degradation and fate of microcystins in lake environments have been proposed. These include: hydrolysis, bacterial degradation (biotransformation), photolysis and sorption. In the absence of bacteria or photosynthetic pigments, microcystins are quite stable in water, undergoing only slight hydrolysis over time. For example, Wannamacher (1989) found that MC-LR degraded by 0.37% d⁻¹ at 45 °C and negligibly at 4 °C. Similarly, Lam et al.

(1995a) report negligible or inconsistent degradation of MC-LR in sterile control flasks during several biotransformation experiments. MC-LR does however, undergo biodegradation in the presence of bacteria. Lam et al. (1995a) found substantial degradation of MC-LR ($T_{1/2}$ =0.2 to 3.6 d) when exposed to effluent from a wastewater treatment plant, but only after an initial lag period of 7 to 14 d. This lag time likely reflected an adaptation period when the bacteria were generating enzymes to degrade the microcystin. Similarly, Jones et al. (1994) observed a substantial decrease (from 1 mg L^{-1} to $< 50 \text{ } \mu\text{g L}^{-1}$) in MC-LR concentrations in flasks inoculated with raw river water over 14 d. In contrast, Kiviranta et al. (1991a) detected no biodegradation of [D-Asp³]MC-RR in cultures of *O. agardhii* over a 90-d period, inoculated with raw river water.

Photolysis has been reported to be a possible pathway of degradation for cyanobacterial hepatotoxins. Tsuji et al. (1994) found that while MC-LR was not degraded by sunlight alone, substantial degradation, and also isomerization to [6(z)Adda⁵]MC-LR could occur in the presence of photosynthetic pigments. The conversion of MC-LR to the non-toxic, 6(z)Adda⁵ analogue was reversible. The non-toxic isomer therefore was freely converted back to the toxic parent form. The rate of degradation was dependent on pigment type and concentration in the water. In one experiment Tsuji et al. (1994) found less than 25% degradation of MC-LR over 15 d when the chlorophyll *a* concentration was 20 mg L^{-1} and almost 50% degradation when in the presence of water-extractable pigment (mainly phycocyanins; $5,000 \text{ mg L}^{-1}$) over the same time period. In another trial examining the effect of pigment concentration on MC-LR degradation, almost 100% of the toxin degraded at a water-extractable pigment concentration of $5,000 \text{ mg L}^{-1}$, but less than 20% degraded when in the presence of 5 mg L^{-1} over a longer 29-d period. MC-RR and -YR also behaved similarly to MC-LR in their experiment. Therefore, even at the lowest pigment concentration (a pigment concentration far exceeding that which is found typically in hypereutrophic lakes in Alberta), photolytic degradation of microcystins would take up to several months. This is an unacceptably long period of time for a drinking water supply.

Given the hydrophobic nature of part of the microcystin structure, sorption to suspended materials (clay micelles, organic suspended particulates and sediments, etc.) has been hypothesized as a natural pathway of microcystin removal from water. Lam and Prepas (1995) however, found little sorption of MC-LR to sediments collected from hypereutrophic lakes in Alberta. Given the stability of microcystins in the above studies, taking a domestic water supply off-line for several weeks to allow for the degradation of microcystins may be an unrealistic or unacceptable solution, especially in water supplies where toxic blooms are noted frequently or continually over a summer.

A common misconception about cyanobacterial toxin stability is that boiling of water containing algal toxins will deactivate or destroy the toxins. Wannamacher (1989) found that autoclaving MC-LR in water destroyed less than 50% of the microcystin. Thus boiling water containing microcystin is not considered a safe means of treating microcystin-contaminated water.

Microcystins are also very stable in acidic and basic conditions. Berg and Soli (1985a) found no reduction in the toxicity of MC-LR at pH 2 or 12, and Wannamacher (1989) found only 20% deactivation of MC-LR when exposed to either 1.0N NaOH or 2.5N HCl. Microcystins are therefore stable in the acidic environment of the gastrointestinal tract of animals and in the presence of enzymes found within the gastrointestinal tract of a wide variety of animals (Botes et al. 1982b; Falconer et al. 1986). The inability of gastric and intestinal enzymes to destroy microcystins likely lies in the cyclic structure of the toxins and in the unusual combination of L and D amino acids.

Most conventional water treatment practices do not remove microcystins from drinking water adequately. Experiments to determine the efficiency of various treatments to remove microcystins from drinking water have been conducted with dissolved toxin, cyanobacterial extracts containing microcystins as well as intact cyanobacterial cells. These studies have utilized experimental designs ranging from simple flask experiments to more complex and realistic pilot plant studies. Few studies have evaluated the *in situ* efficiency of operational water treatment facilities (Lambert et al. 1994a).

Flocculation, sand filtration and chlorination is the most commonly used treatment practice, but removes little microcystin from drinking water (usually less than 20 to 30%

of the initial concentration; Wheeler et al. 1942; Hoffman 1976; Keijola et al. 1988; Himberg et al. 1989). Flocculation steps that include lime or alum do little to remove microcystins from the water unless the toxins are still within the cells (Lam et al. 1995b). Ferric chloride is also used as a flocculating agent. Himberg et al. (1989) showed that ferric chloride may actually release microcystin from the cells into the water as does odor-controlling agents such as potassium permanganate (Lam et al. 1995b). Powdered activated carbon (PAC) is widely used as a flocculant but can also be effective at removing dissolved microcystins from water (Falconer et al. 1983b, 1989; Keijola et al. 1988; Jones et al. 1993; Donati et al. 1994), although high doses of carbon are required for complete removal. In practice however, the use of PAC may not always be justified. For example, Jones et al. (1993) found that to bring microcystin concentrations below their target level ($<1 \mu\text{g L}^{-1}$), high PAC doses (up to 100 mg L^{-1}) and long contact times (up to 120 min) were needed after the flocculation step. The high PAC doses required could be prohibitively expensive (a treatment plant with a throughput of 100 ML d^{-1} applying PAC at 50 mg L^{-1} would consume $5 \text{ tonnes of PAC d}^{-1}$). At $\$3,000 \text{ tonne}^{-1}$, this was considered unreasonable (Jones et al. 1993). Additionally, high PAC doses may significantly decrease filtration efficiency. The capacity of PACs to adsorb microcystins is also dependent on the type of carbon used. Donati et al. (1994) found that wood-based carbons were more effective at removing dissolved MC-LR than coconut and peat moss-based carbons. The differences in microcystin-adsorbing properties reflected differences in mesopore volume between the PACs evaluated. Competition by natural organic matter in water may also reduce the adsorption efficiency of PACs for MC-LR, by up to 52% (Donati et al. 1994).

Water treatment trains that include granular activated carbon filtration and/or ozonation are effective at removing or destroying microcystins to non-detectable levels (Keijola et al. 1988; Himberg et al. 1989; Jones et al. 1993) depending on contact time and initial microcystin concentration. Disinfection, the last step in water treatment, usually involves chlorination. Chlorination of water containing microcystins has generally been shown to have a negligible effect on microcystin concentration (Hoffman 1976; Keijola et al. 1988; Himberg et al. 1989). However, Nicholson et al. (1994) recently showed that aqueous chlorine ($1 \text{ to } 2 \text{ mg L}^{-1}$) and calcium hypochlorite ($2.5 \text{ to } 16 \text{ mg L}^{-1}$) destroyed

90% of the MC-LR with a contact time of 30 min. Sodium hypochlorite (15 to 30 mg L⁻¹) achieved 50% removal in 30 min. In contrast, monochloramine (20 mg L⁻¹) was ineffective at destroying MC-LR after a 5-d contact time. I would suspect that chlorination of microcystin-containing water in Alberta may destroy little microcystin. Firstly, pH of water in many drinking water sources likely to be contaminated with toxin-producing cyanobacteria would be fairly high during the summer (8 to 10), thus making it difficult to maintain an effective chlorine residual. In addition, high organic loads in these drinking water sources would react quickly with chlorine to form chloramine, which is not as strong an oxidant as chlorine. Therefore the chlorine would be consumed by the organic load before it has a chance to destroy the microcystins.

It is important to note that many of the initial experiments that examined the effectiveness of various water treatment practices at removing microcystins, used mouse bioassays to detect toxin concentrations in the water. Given that the detection limit by mouse bioassay is much higher than by instrumental analysis (e.g., HPLC), the complete loss of mouse toxicity from a water sample after a treatment step does not imply that microcystins were completely removed or destroyed. Indeed, a similar problem is also evident in many of the more recent publications that utilized HPLC to analyze water samples. In these cases, results are commonly reported as % microcystin removal. Even at a seemingly impressive removal of 90%, a substantial amount of microcystin may still be present in the water since most of these studies utilized high microcystin concentrations to ensure adequate detection by HPLC. Jones et al. (1993) recognized this problem and suggested that actual concentrations be reported. They also used a realistic target concentration (1 µg L⁻¹) in their experiments to determine if a water treatment step was effective. This concentration is also the approximate value that some countries are considering as a guidance value for microcystins in drinking water.

It has been a common belief in the water treatment industry that the absence of odors caused by cyanobacteria implies that toxins such as MC-LR will also be absent. There has been no evidence to date to show a relationship between the concentrations of geosmin or MIB and MC-LR in phytoplankton (Kenefick et al. 1992; Hrudefy et al. 1993).

Therefore, the absence of musty odors should not be regarded as a sign that the phytoplankton will not contain MC-LR.

3.2 Nodularins - The first published report linking animal poisonings to the accidental ingestion of water containing cyanobacterial toxins (Francis, 1878) involved the brackish water cyanobacterium, *Nodularia spumigena*. *N. spumigena* is now known to produce several monocyclic pentapeptides, termed nodularins, that have hepatotoxic properties. Nodularins have also recently been isolated from the marine sponge *Theonella swinhoei* (deSilva et al. 1992), speculated to be produced by a microbial symbiont rather than the sponge itself (Rinehart et al. 1994). The basic nodularin structure is very similar to microcystins except that it is comprised of only five amino acids; it lacks one of the D and L amino acids of the microcystins (Fig. 5). Nodularin has the structure cyclo (-D-MeAsp-L-Arg-Adda-D-Glu-Mdhb-) where Mdhb is N-methyl-dehydrobutyric acid (Carmichael et al. 1988b; Rinehart et al. 1988; Sivonen et al. 1989b). This is the only cyanobacterial hepatotoxin that contains Mdhb; none of the microcystins contain this amino acid. Variations on the basic nodularin structure include substitution of arginine with valine (giving [L-Val²]nodularin, also termed motuporin) demethylation of the aspartic acid and ADDA residues (giving [D-Asp¹]nodularin and [DMAdda³]nodularin, respectively), a methylester of the Glu and MeAsp acid residues (giving [D-MeAsp(OCH₃)¹, D-Glu(OCH₃)⁴]nodularin) and a geometric isomer of Adda (giving [6(z)Adda³]nodularin; deSilva et al. 1992; Rinehart et al. 1994). While nodularin has an LD₅₀ of 50 µg kg⁻¹ in mice (by intraperitoneal injection), the geometric isomer and the dimethylester analogues are not toxic to mice (Rinehart et al. 1994). The two demethylated analogues, [D-Asp¹]nodularin and [DMAdda³]nodularin, have an LD₅₀ of 75 and 150 µg kg⁻¹, respectively (Table 2). In addition, Choi et al. (1993) have isolated four linear peptides from nodularin and microcystin-containing samples. These compounds may be precursors to their cyclic toxin counterparts. In all cases the linear compounds were non-toxic, suggesting that the cyclic nature of the compounds confers toxicity. No systematic studies have evaluated the stability and chemical properties of nodularins. However, given its similar structure to the microcystins, one would also suspect similarity to the microcystins in terms of water solubility, hydrolytic and enzymatic

stability, photodegradative properties, and biodegradation potential. The extraction procedures, solvents and conditions of HPLC analysis are very similar to that of the microcystins, further support for similar structures.

4. Detection and Quantification of Microcystins

The origins of detection methods for microcystins, and indeed all freshwater and marine phycotoxins, can be traced to the mouse bioassay method. In the early part of this century, the toxicity of cyanobacterial blooms suspected of killing animals was assessed by injection of cyanobacterial cell extracts into small rodents (usually mice). This method is still very much used today as a toxicity screening tool. Beginning in the early 1980s, coincident with the structural determination of five microcystins, more sophisticated methods of detection were developed. These include other types of biological assays as well as analytical methods, including high performance liquid chromatography (HPLC).

4.1 Mouse and other animal bioassays - The mouse bioassay is still the mainstay of cyanobacterial toxicity testing. Although there are many other methods which are much more sensitive, the mouse bioassay is still used worldwide as either an initial toxicity screening tool, or in some studies, as the sole method of detection (Carmichael et al. 1988c; Lanaras et al. 1989; Sivonen et al. 1989b, 1990b; Repavich et al. 1990). The mouse bioassay involves extraction of a known mass of cyanobacteria in physiological saline followed by intraperitoneal injection of the extract (less than 1 mL) into a series of mice. The method can give either qualitative or semi-quantitative results, depending on how the bioassays are run.

Under the qualitative regime, one or two mice are injected with the same mass of cell extract and the responses of the mice are assessed: whether the animals die within 24 h, whether the animals display clinical signs of poisoning (pallor of the eyes, ears and tail, in the case of microcystins, gasping for breath, convulsions, respiratory arrest in the case of neurotoxins), and gross pathology of the liver (color of the liver and an increase in the mass of the liver relative to the body, also in the case of microcystins). The toxicity of the cyanobacterial sample is then scored as toxic (the mice died), slightly toxic (either the mice displayed clinical signs of poisoning, gross pathology to the liver, or both) or non-toxic

(no visible effect on the mice). The detection limit is both dependent on the sensitivity of the individual animals (which is influenced by size, gender, whether the animal is fed or fasted) and the mass of cyanobacterial extract injected (Carmichael 1988). Generally less than 50 mg of cells is injected into the animals to avoid secondary effects caused by the extract. Semi-quantitative results can also be obtained with the mouse bioassay. In this case, groups of mice are injected with a different mass of cells, producing an LD₅₀ trial. The LD₅₀ value, expressed as either mg cells kg⁻¹, or as the ill-defined and rarely-used mouse unit (the minimum dose required to kill a 20 g mouse), can then be compared with those for other cyanobacterial samples.

The mouse bioassay has some advantages over other more contemporary and sophisticated methods of detection, thus contributing to its continued use worldwide. The bioassay is relatively inexpensive compared to analytical detection methods (such as HPLC), is quick to perform and can give a direct estimate of animal toxicity. However, in some countries, many have moved away from the use of such biological assays, for both scientific and ethical reasons. One major disadvantage of the mouse bioassay is the poor detection limits. The method can detect toxin concentrations in the cyanobacteria that may cause acute animal poisonings, but is not sensitive enough to detect even high concentrations of the toxins dissolved in water and those that may represent a chronic health risk. For example, a 20 to 30 g mouse can be injected with 25 mg of cell extract without causing secondary affects to the animal. The minimum toxin concentration in the cells that could be detected (i.e., the minimum concentration causing death) is approximately 1 µg for MC-LR, or about 40 µg g⁻¹ of cells. In addition, the mouse bioassay method would only be able to detect dissolved MC-LR in a drinking water sample containing more than 1,000 µg L⁻¹ (assuming the toxin was not preconcentrated prior to injection and that the mouse was injected with a volume of 1 mL). Another disadvantage of the mouse bioassay is that it cannot distinguish between the more than 50 microcystin analogues that may be present in the sample. The presence of cyanobacterial neurotoxins in the sample may also completely mask the presence of the hepatotoxins. Because the neurotoxins can kill the mouse rapidly (5 to 10 min after intraperitoneal injection) the clinical signs of poisoning and pathology to the liver by the hepatotoxins

would not be evident. Finally, there are also ethical concerns related to the use of animals for monitoring programs for toxic compounds.

In addition to mice, other small mammals have been used for toxicity testing of cyanobacterial samples. These include rats, hamsters, rabbits and guinea pigs (Schwimmer and Schwimmer 1968). Recently, Kiviranta et al. (1991b) examined the utility of brine shrimp larvae (*Artemia salina*) as a alternative whole-animal toxicity screen. Although they found good agreement between the levels of toxicity of cyanobacterial bloom samples and laboratory strains of cyanobacteria by mouse bioassay, with that determined by *A. salina* bioassay, the detection limit was low, comparable to the mouse bioassay. In addition, the shrimp bioassay failed to detect toxicity in some samples when the samples were toxic by mouse bioassay (although the toxicity was low in the mouse bioassay) and gave positive toxicity results in other samples where mouse toxicity was not detected (Kiviranta et al. 1991b). Kiviranta et al. (in press) also examined the utility of mosquito larvae (*Aedes aegypti*) as a screening tool and also suggested that because of the high toxicity of the cyanobacteria to the mosquito larvae, biological control of mosquito populations in tropical countries via toxic cyanobacteria may be possible.

The use of luminescent bacteria as an indication of the toxicity of environmental samples (i.e., the Microtox Assay) has increased dramatically in the last decade (Bulich 1986). Lawton et al. (1990) investigated the utility of Microtox as a means of screening cyanobacterial samples for toxicity. They found that while the assay generally gave comparable results to the LD₅₀ values generated from mouse bioassays, there were some discrepancies. Since Lawton et al. (1990) compared Microtox only to mouse bioassay, it is not known how the detection limit compares with other methods, such as HPLC.

4.2 Animal cell and tissue bioassays - Animal cell and tissue bioassays for the detection of microcystins have several advantages over whole animal bioassays. Treatments can be easily replicated, the bioassays are relatively inexpensive and quick to run and large laboratory space is not required. As with whole animal bioassays however, the cell and tissue assays cannot distinguish between microcystin analogues. Additionally, animals are needed to establish the cell or tissue culture lines. In some cases, the cells must be prepared fresh each time (Aune and Berg 1986). Cell bioassays that have been

used for microcystin detection include red blood cells (Carmichael and Bent 1981) and freshly isolated rat liver cells (Aune and Berg 1986; Berg and Aune 1987). In addition, cultures of hamster lung fibroblasts have also been used (Codd et al. 1989b).

Carmichael and Bent (1981) evaluated the utility of human red blood cells for the detection of hepatotoxins and neurotoxins in cyanobacterial samples. Although hemagglutination of the red blood cells was associated with mouse toxicity, the mechanism(s) causing the agglutination were not identified. The method only allowed for qualitative comparisons between samples, and has not been mentioned in the literature since.

Freshly isolated rat hepatocytes have also been used as a screening tool for cyanobacterial hepatotoxins. To assess toxicity, Aune and Berg (1986) and Berg and Aune (1987) scored the number of deformed hepatocytes after varying degrees of incubation time and also the release of lactate dehydrogenase (LDH) from the cells. These parameters of toxicity correlated well with results from mouse bioassays on the same samples. One advantage of this method over mouse bioassays is that one rat liver provided enough cell material to run 10 to 15 cyanobacterial cell samples in triplicate. However, a facility supporting animal surgery is still required as the liver must be perfused with collagenase *in situ* to dissociate the hepatocytes and maintain hepatocyte viability. The method would also not be able to detect neurotoxins. Similarly, Codd et al. (1989b) used Chinese hamster lung fibroblast cultures to assess the toxicity of cyanobacterial samples. The assay was based on reduction of protein production by the fibroblasts in the presence of the cyanobacterial extracts.

4.3 Instrumental methods of identification and quantification - Instrumental methods of analysis for microcystins have three objectives: separation of the toxin(s), identification and quantification. Separation methods have included HPLC (Namikoshi et al. 1992d; Craig et al. 1993), high performance thin layer chromatography (HPTLC, Poon et al. 1987; Al-Layl et al. 1988; Codd et al. 1989b) and thin layer chromatography (TLC, Ojanpera et al. 1991) while structural identification of the isolated toxins has been determined with amino acid analysis of the hydrolyzed peptides by HPLC (Harada et al. 1990a; Kiviranta et al. 1992), various mass spectroscopy methods including fast atom

bombardment-mass spectroscopy (FAB-MS) and high resolution FAB-MS (HRFAB-MS) (Namikoshi et al. 1990; Kiviranta et al. 1992; Namikoshi et al. 1992d), plasma desorption-MS (Craig et al. 1993) and tandem FAB-MS/CID-MS (collisionary induced decomposition-MS; Rinehart et al. 1994), and by ^1H nuclear magnetic resonance analysis (^1H -NMR, Harada et al. 1990a; Sivonen et al. 1990a; Kiviranta et al. 1992)

The most common instrumental method for routine microcystin analysis however, is separation by high performance liquid chromatography (HPLC) with UV detection at 238 nm (Meriluoto et al. 1990a; Harada et al. 1991a+b; Kenefick et al. 1992; Namikoshi et al. 1992d; Sivonen 1990). Reverse phase column packings (such as C-18) appear to be the most widely used column material, although some have successfully used weak anion exchange columns (Gathercole and Theil 1987). The separation of analytes by reverse phase HPLC is based on three mechanisms: 1) molecular size, with smaller molecules penetrating the pores in the column packing material and thus eluting from the column later than larger molecules, 2) hydrophobic interactions with the column packing material, with more hydrophobic compounds or those with hydrophobic side chains interacting the most and therefore eluting later than more hydrophilic compounds, and 3) interaction with the mobile phase.

Analysis of microcystins by HPLC involves extraction of a sample (phytoplankton or laboratory culture) in a suitable solvent (usually water, methanol or acetic acid, although combinations of water-butanol-methanol have been used). The microcystins in the sample are then partially purified and concentrated by solid phase extraction with disposable C-18 cartridges. Some of the compounds that may interfere with the HPLC analysis are sequentially removed from the cartridge by use of solvents of differing ionic strength (water, 5% acetic acid, 10% methanol in water). The microcystins are then eluted from the cartridge in 100% methanol and injected onto the HPLC column. The mobile phases (consisting of an aqueous and organic solvent) most often used for separation and quantification include ammonium acetate with acetonitrile (Sivonen et al. 1990a; Kenefick et al. 1992), potassium dihydrogenphosphate and acetonitrile (Meriluoto et al. 1990a) or potassium dihydrogenphosphate and methanol (Watanabe et al. 1989). The choice of which type of mobile phase to use, and whether additives such as TFA

(Craig et al. 1993; Lawton et al. 1994) are used, appears to be based on personal preferences of the investigator, and the HPLC system used. For example Kenefick (1991) found that the use of phosphate salt buffers may accumulate and block the flow cells in some detectors. All of the mobile phases mentioned, and a multitude of variations of these, are often equally efficient at separating microcystin analogues from each other and from other compounds.

Isocratic separation is the most commonly used HPLC separation method for routine monitoring of microcystins by HPLC. Isocratic separation involves injecting a cyanobacterial extract into a mobile phase in which the proportion of the aqueous and organic solvents (ammonium acetate and acetonitrile, for example) is held constant throughout the duration of the run. Until very recently, gradient separations (when the proportion of the two solvents in the mobile phase is changed throughout the run) were utilized only for isolating microcystins for structural analysis. However, the use of gradient separation is becoming more widespread for routine microcystin analysis, possibly a result of programmable HPLC systems which now allow for user control of the rate of solvent change as well as the shape of the gradient. Lawton et al. (1994) developed a gradient method based on a mobile phase of water-TFA and acetonitrile-TFA which they suggest can adequately separate up to 10 microcystins in cyanobacterial and water samples. I have also developed a gradient method based on a mobile phase of ammonium acetate and acetonitrile, which can successfully separate MC-LR from other compounds in complex matrices such as cyanobacteria and invertebrate tissues (e.g., zooplankton; see Chapter 6). The gradient method gave much better separation of MC-LR from other peaks than an isocratic method with the same mobile phase (ammonium acetate:acetonitrile, 80:20%, v/v). For example, Fig 6 shows the chromatograms of a phytoplankton sample from Driedmeat Lake, collected on 11 August 1994, analyzed by HPLC with an isocratic separation (10mM ammonium acetate:acetonitrile, 80:20%, v/v; Fig. 6a) and by gradient separation (20-30% acetonitrile in 10mM ammonium acetate over 10 min followed by 20% until the end of the run; Fig. 6b). The gradient method resolved MC-LR from the other peaks much better and also resolved many other peaks in the sample, peaks that were lumped together and thus masked in the isocratic method.

Despite its widespread use in microcystin analysis, HPLC does have its limitations. For example, standards for the majority of the 50 plus microcystins are not available commercially. Investigators must therefore rely on the generous gifts of fellow researchers. This may put a severe limit on studies, such as those which involve large quantities of toxin. Only three microcystin standards (-LR, -RR and -YR) and one nodularin analogue are available commercially in North America. Lawton et al. (1994) have partially overcome this hindrance by utilizing photodiode array detection (UV detection that is able to scan absorbance not just at 238 nm, but over a range of wavelengths). Most microcystins have a characteristic UV spectrum with a strong peak in absorbance at 238-240 nm, with the exception of tryptophan-containing analogues. By using photodiode array detection, the UV spectrum of individual peaks in a sample run can be made. Lawton et al. (1994) claim that the absorption spectra of the peaks can be used to assigned the peaks to particular microcystin analogues with a high degree of confidence. However, since the response of the detector to the different microcystin standards varied, each microcystin had a slightly different calibration curve. Therefore, while photodiode array detection alleviates the need for many microcystin standards for tentative identification, it does not eliminate the need for individual standards for quantification. Standards for individual microcystins should still be used to increase the confidence in peak identification. However, given the severe lack of analytical standards, some researchers are currently generating calibration curves based on one microcystin standard (e.g., MC-LR) to quantify other microcystins identified by diode array detection in phytoplankton samples (G. Quibell, pers. comm., State Water Authority, Pretoria, South Africa).

While the detection limits reported for HPLC methods are much better than traditional methods such as mouse bioassays, they may not be sufficient for analyses that require detection of trace quantities of microcystins, such as monitoring concentrations in drinking water. HPLC can be up to 100 times more sensitive than mouse bioassay. For example, my detection limit for purified MC-LR by the HPLC gradient method described previously (e.g., 20 to 30% acetonitrile in 10 min), was approximately 1 ng per 20 μ L injection (Fig. 7a). However, interference caused by co-extractives in cyanobacterial

sample matrices tended to increase the detection limit slightly; 10 to 20 ng in the cyanobacterial extracts per 20 μ L injection (Figs. 7b, 8a+b). In contrast, the minimum MC-LR concentration that can be detected by mouse bioassay is generally 1 μ g in a 1 mL intraperitoneal injection. The limit to detecting lower concentrations of microcystins by HPLC is the method of detection. We have pushed the envelop of detection by UV absorbance. With purified microcystin standards, UV detection is not able to be lowered much below about 1 ng in a 20 μ L injection (see Fig. 7a) due to limitations imposed by the UV detector. Two biological methods hold promise for detection of trace quantities of microcystins in a variety of sample matrices (water, cyanobacteria, tissues): immunoassay and protein phosphatase bioassay.

4.4 Immunoassay - Immunoassays for the detection of microcystins have been developed with both mono and polyclonal antibodies. In a direct competitive immunoassay such as the Enzyme-Linked Immunosorbent Assay (ELISA), small animals, such as rabbits, are injected with a microcystin standard (usually MC-LR that has been previously conjugated to other compounds, such as bovine serum albumin (BSA)). This conjugation step is necessary as microcystins are too small by themselves to elicit an immunological response from the animals, and given the toxicity of the microcystins, even injections of small quantities of the toxins could kill the animals. The polyclonal antibodies (anti MC-LR antibodies) are then isolated from the rabbits and purified, and coated onto microtiter plates. A small volume of sample (water, cyanobacterial extract) is then added to the plate along with MC-LR that has been previously conjugated to horseradish peroxidase (HRP). Cross-reaction of microcystin-HRP with the anti-microcystin antibodies coated on the plate, produces a color change (in the presence of required reagents) which can be quantified spectrophotometrically. The rate of change in the color formation is a function of the amount of microcystin(s) in the sample added, since microcystins in the sample will compete with microcystin-HRP in the assay for binding sites on the anti-microcystin antibodies. Thus this form of ELISA is known as direct competitive ELISA (Chu et al. 1990).

For the ELISA to be a useful monitoring tool, it must fulfill two requirements: low detection limits and the ability to detect a large number of microcystin analogues. The

success of the first attempts at developing immunoassays for microcystins were mixed. Detection limits were not much better than instrumental methods such as HPLC. Standard calibration curves reported by Kfir et al. (1986) and Brooks and Codd (1988) were in the microgram range. Modification to the original methods by Chu et al. (1990) has pushed the envelop of detection into the pg range (25-500 pg assay⁻¹) and calibration curves reported by An and Carmichael (1994), with an IC₅₀ of 3.1 ng mL⁻¹ for MC-LR, should allow for the detection of trace quantities (ng g⁻¹) of microcystins in tissue samples.

Immunoassays, such as ELISA, cannot distinguish between the various microcystin analogues. The final concentration reported, under ideal conditions, would represent total microcystin concentration. However, many of the microcystin analogues may not cross react with the anti MC-LR antibodies. Thus these microcystins would go undetected and the total microcystin pool would be underestimated. For example, Chu et al. (1990) reported poor cross-reactivity of MC-LY and -LA with anti MC-LR antibodies, and An and Carmichael (1994) reported poor cross-reactivity of microcystins which contain demethylated Adda components (e.g., [DMAdda⁵]MC-LR). These microcystin analogues however, are toxic in the mouse bioassay. Therefore, some microcystins that are toxic would not be detected in ELISA. Additionally, it is likely that other microcystin analogues that are non-toxic by mouse bioassay, would be detected by ELISA. For example, An and Carmichael (1994) reported good cross-reactivity of a microcystin which had a monoester modification to the toxin (i.e., [D-Glu-OCH₃⁶]MC-LR). Therefore this non-toxic microcystin would be included in the microcystin pool detected by ELISA.

4.5 Protein phosphatase bioassay - One of the most promising biological assays for microcystin analysis is the protein phosphatase bioassay. This method of detection is based on the actual mode of action of the microcystins: the inhibition of the enzymes protein phosphatase 1 and 2a in eukaryotic cells (MacKintosh et al. 1990). In eukaryotic cells, protein phosphatases cause the dephosphorylation of phosphorylase a to phosphorylase b (discussed later). In the bioassay, phosphorylase a is radio-labeled with ³²P. In the absence of inhibitors (such as microcystins and nodularins), ³²P is liberated from phosphorylase a by a protein phosphatase (e.g., PP1c) in the assay mixture. The radioactivity is then determined by liquid scintillation. In the presence of a sample

containing microcystins, this reaction is inhibited in a concentration-dependent manner, and thus the amount of ^{32}P liberated is decreased. The reduction in liberated ^{32}P can be compared to that caused by MC-LR standard (i.e., a calibration curve). Because the reduction in radioactivity with increasing MC-LR concentration is linear only over a small range of toxin concentration, the samples usually have to be diluted to bring the inhibition into the linear portion of the calibration curve to get accurate quantitation. The detection limit is comparable to ELISA, with detection in the low pg range. Also like ELISA however, the method cannot distinguish between the various microcystin analogues.

To alleviate the problems of not being able to distinguish toxic analogues in samples, Boland et al. (1993) developed a novel and elegant method termed liquid chromatography-linked protein phosphatase bioassay. In this procedure, a cyanobacterial sample is fractionated by HPLC and the fractions are then analyzed by protein phosphatase bioassay for the ability to inhibit protein phosphatase 1c. The active fractions are then combined and re-chromatographed by HPLC to further separate the active peaks. In this respect, the protein phosphatase assay helps guide the HPLC analysis by indicating which of the peaks are protein phosphatase inhibitors (i.e., microcystins). The active peaks can then be purified and the structures of the toxins determined. This method has been successfully used to identify new microcystin analogues in freshwater cyanobacteria (Boland et al. 1993; Craig et al. 1993), to detect okadaic acid and other diarrhetic shellfish poisons (also protein phosphatase inhibitors) in shellfish (Holmes 1991; Boland et al. 1993) as well as to identify the first known occurrence of microcystins in the marine environment (Andersen et al. 1993; Chen et al. 1993).

The protein phosphatase assay has several advantages over the other methods of detection already described. Detection limits are suitable for detecting trace quantities of the toxins in drinking water samples. Because the assay is based on the mode of action of the microcystins, microcystin analogues that are non-toxic (such as the 6(z) stereoisomers) are not detected in the assay. Therefore, total microcystin concentration would not be overestimated by the presence of these non-toxic forms. Another advantage of the method is that sample preparation is minimal, and in some cases not needed. This is in marked contrast to methods such as HPLC where sample preparation can take a

substantial amount of time. Water samples for example, can be run without any preparation in the protein phosphatase assay. The sample volume of water can also be very small (5 mL), eliminating the need for large volume samples (Lambert et al. 1994a). Cyanobacterial samples can be extracted in a suitable solvent (methanol) and run immediately without the need for sample cleanup steps such as solid phase extraction/separation.

To be accurate at quantifying microcystin concentration in samples, there must be no interference from other non-microcystin compounds in the sample. That is, other protein phosphatase inhibitors must be absent or accounted for. A wide variety of sample types (water, cyanobacteria, invertebrates) have been analyzed by the protein phosphatase method and the HPLC fractions causing inhibition have always corresponded to the class of toxins studied (e.g., okadaic acid, microcystins; C.F.B. Holmes, pers. comm.). Therefore interference by other compounds in the sample matrices may not be a problem. Sim and Mudge (1993) identified another area of concern: the use of the protein phosphatase assay for detecting microcystins in samples that may contain endogenous protein phosphatases (e.g., cyanobacteria, animal tissues). They argued that endogenous protein phosphatases would reduce the amount of microcystin detected in the sample by competing in the reaction mixture for the microcystins in the sample. Holmes (pers. comm.) maintains that if the sample is extracted in 100% methanol, any endogenous protein phosphatase will be inactive, and thus will not compete for microcystins present in the sample.

The protein phosphatase assay has other limitations. The reagents used in the assay can take a considerable amount of time to prepare (Lambert et al. 1994b), and the substrate (phosphorylase a) must be radiolabelled. Protein phosphatase used in the assay was originally obtained and purified from animal tissue (usually rabbit skeletal muscle; Lambert et al. 1994b). Protein phosphatases are now available commercially from Gibco (Life Technology) and Calbiochem. An and Carmichael (1994) have alleviated some of these problems by developing an analysis based on recombinant PP1 expressed in *E. coli* instead of PP1 isolated directly from rabbits, and by using colorimetric methods of

detection instead of ^{32}P . The shift from ^{32}P to colorimetric methods for detection of microcystins will allow more laboratories to utilize protein phosphatase bioassays.

5. The Global Occurrence of Toxic Freshwater Cyanobacteria

The occurrence of toxic blooms of cyanobacteria is not a local, regional or even country-specific phenomenon. Toxic blooms of freshwater cyanobacteria occur all over the globe. Until very recently, many papers provided listings of the countries where toxic blooms have been reported (see Carmichael et al. 1985) to show the widespread occurrence of the toxins globally. I suspect that most if not all eutrophic to hypereutrophic water bodies worldwide will support the growth of neuro- or hepatotoxic cyanobacteria at one time or another, or perhaps on an almost continual basis, as some of my study lakes indicate (see later Chapters of this thesis). The addition of a new country to the list of those already inducted into the "Cyanobacterial Toxin Hall of Fame" will only depend on the happenstance of an animal being at the wrong place and time, similarly for human poisoning or by the chance that a scum sample is collected and examined by a curious layperson or member of the scientific community. Therefore, I believe that it is only a matter of time before most if not all countries are inducted. At present there are no reports of toxic cyanobacteria from polar regions. It would be interesting to determine if toxin-producing cyanobacteria are present in lakes and ponds located in arctic and subarctic regions during the ice-free season. Reports of toxic blooms during winter months in ice-free lakes in the State of Washington, U.S.A (Jacoby et al. 1994), and in ice-covered lakes in Finland during the winter (J.E. Eriksson, pers. comm.) goes against the common dogma which dictates that toxic production is restricted to warm (15 to 30 °C) water. Indeed, it is very likely that toxin-producing cyanobacteria are much more prevalent than is currently surmised based on animal poisonings alone.

The routine monitoring of the toxicity of blooms or the identification of blooms as being toxic or non-toxic has generally been based on either animal poisonings or by use of mouse bioassays. Examples of routine monitoring by instrumental methods of detection (e.g., HPLC) are rare, and by methods such as ELISA or protein phosphatase, even rarer.

5.1 *Toxic bloom occurrence based on animal poisonings* - Schwimmer and Schwimmer (1968) have summarized most of the known incidents of animal poisonings by freshwater cyanobacteria from the time of George Francis (1878) to the early 1960s. Since Schwimmer and Schwimmer's summary, numerous reports of animal poisonings have been published including dogs (Elder et al. 1985; Edwards et al. 1992), livestock (Main et al. 1977; Beasley et al. 1983; Galey et al. 1987; Kerr et al. 1987; Chengappa et al. 1989) and wildlife (Soll and Williams 1985; Pybus et al. 1986). The number of published accounts of animal deaths and poisonings attributed to toxic cyanobacteria has decreased substantially in the last five to ten years, possibly because these incidents are no longer considered novel. Given the large number of eutrophic to hypereutrophic lakes on the Canadian prairies, one should also expect published accounts of similar animal poisonings from this region of Canada. Table 3 summarizes all of the published accounts I could find on animal poisonings in Canada that were attributed to toxic cyanobacteria. Many more are likely archived in government reports, veterinary records, and in personal memoirs. Most cases of poisonings involved the three most commonly reported cyanobacterial offenders: *Anabaena* spp., *Aphanizomenon* spp. and *Microcystis* spp. (known affectionately to phycologists as Annie, Fanny and Mike, respectively). The suspected toxins were likely anatoxins, PSPs and microcystins (Table 3).

5.2 *Cyanobacterial toxin monitoring based on mouse bioassays* - Monitoring for cyanobacterial toxins has traditionally been done by examining a wide range of lakes on a one time basis. Few studies have monitored toxin concentrations in a lake over time, unless animal poisonings or fatalities occur repeatedly at the lake. The determination of whether phytoplankton from a lake is toxic or not has traditionally been evaluated by mouse bioassays. Studies that have screened the largest number of lakes for toxic cyanobacteria by the mouse bioassay include Sivonen et al. (1990b; 188 sites in Finland), Berg et al. (1986; 51 sites in Finland, Sweden and Norway), Codd et al. (1989a; 24 sites in the UK), Lawton and Codd (1991; 91 sites in the UK) and Repavich et al. (1990; 102 sites in Wisconsin, U.S.A.). On average, 54% of the sites evaluated in the above studies were either hepatotoxic or neurotoxic to mice, with a range of 25% (Wisconsin) to 75% (UK) of samples being toxic. Despite the much better detection limits offered by instrumental

methods of analysis (such as HPLC) monitoring for cyanobacterial toxins still relies heavily on the mouse bioassay (Carmichael et al. 1988c; Lanaras et al. 1989; Sivonen et al. 1989b, 1990b; Repavich et al. 1990), possibly due to the lower cost relative to HPLC.

5.3 Cyanobacterial toxin monitoring based on instrumental analysis - Few attempts have been made to evaluate cyanobacterial toxin concentrations in natural phytoplankton samples from a large number of lakes in a region, or in a single lake over time by instrumental analysis. Studies focused on microcystins have included Scott's (1991) examination of phytoplankton collected from 5 regions of southern Africa for 6 microcystin analogues, a study by Wicks and Thiel (1990) who examined seasonal changes in the production of six microcystin analogues in Hartbeespoort Dam, South Africa over 2.5 yr, and seasonal changes in 3 microcystin analogues (-RR, -YR and -LR) in Japanese lakes (Watanabe et al. 1989; Shirai et al. 1991). In addition, Sivonen et al. (1989a) quantified anatoxin-a concentrations by gas chromatography-mass spectrometry (GC-MS) in 30 phytoplankton samples collected from several regions of Finland. In the above studies monitoring microcystins, all samples examined contained detectable concentrations of at least one microcystin analogue. In most cases, more than one analogue was present. In addition, Sivonen et al. (1989a) found that 43% of the samples examined, had detectable anatoxin-a concentrations. During the period from 1990 to 1994, I collected phytoplankton from 28 lakes and 11 farm dugouts in Alberta and 3 storm water retention ponds located in Edmonton, which were analyzed for MC-LR by HPLC. Some water bodies were sampled once, while others were sampled extensively (>100 times) from 1990 to 1994 (Fig. 9a). I found that MC-LR was present in a high proportion of the lakes sampled. Twenty four of the 28 lakes (i.e., 86%) contained detectable concentrations (>1 $\mu\text{g g}^{-1}$) of MC-LR at least once (Fig. 9a). MC-LR was also detected in phytoplankton collected from 6 farm dugouts and one storm water retention pond. In the case of the lakes, only eutrophic to hypereutrophic lakes (i.e., lakes with high summer phosphorus concentrations or cyanobacterial biomass) were sampled. Few mesotrophic or oligotrophic lakes were surveyed. In total, 490 bloom samples were collected and analyzed from the lakes, 360 (73%) of which contained MC-LR. This was a surprisingly high incidence of occurrence. The mean concentration of MC-LR (average of all data

collected from 1990-1994) in the lakes was highest in Little Beaver and Driedmeat lakes (504 and 322 $\mu\text{g g}^{-1}$, respectively; Fig. 9b). In Little Beaver Lake, MC-LR concentration ranged from non-detectable to over 1,500 $\mu\text{g g}^{-1}$ over the five years of study. Other lakes such as Beaverhill, Narrow, Lesser Slave and Wabamun had no detectable concentration of MC-LR (Fig. 9b), although several of these lakes were sampled only once (Fig. 9a) and thus the single sample may not be representative for those lakes. The high percentage of blooms containing microcystins by HPLC compared to those by mouse bioassay likely reflects the much better detection limits of HPLC.

5.4 Microcystin monitoring based on ELISA and protein phosphatase bioassay -

Only two studies have reported microcystin concentrations in phytoplankton and lake water by ELISA and protein phosphatase bioassay detection, likely a result of the lack of availability of these relatively new methods. Chu et al. (1990) reported total microcystin concentrations of 26 to 5,200 $\mu\text{g g}^{-1}$ in phytoplankton and from 0.006 to 2.9 $\mu\text{g L}^{-1}$ in lake water from samples collected in Wisconsin. Lambert et al. (1994a) used protein phosphatase bioassay to estimate dissolved microcystin concentrations in raw and treated lake water collected from Driedmeat and Little Beaver lakes, Alberta in 1992. Total microcystin concentrations (expressed as MC-LR equivalents) in raw lake water ranged from 0.15 to 0.87 $\mu\text{g L}^{-1}$ while concentrations in treated drinking water ranged from 0.09 to 0.18 $\mu\text{g L}^{-1}$.

6. Variability of Toxin Concentrations in Phytoplankton Assemblages

Studies which have monitored the toxicity of phytoplankton assemblages or the actual concentration of the toxins in the phytoplankton, by mouse bioassay and HPLC respectively, have demonstrated that toxicity/toxin concentration can vary substantially within one lake on a particular day (Carmichael and Gorham 1981), within a year (Lanaras et al. 1989; Wicks and Thiel 1990) and between years in the same lake (Sivonen et al. 1989a; Watanabe et al. 1989; Wicks and Thiel 1990; Shirai et al. 1991). This variability can be ascribed to at least four major factors: 1) phytoplankton dynamics (changes in the proportion of toxic and non-toxic species), 2) the variable presence of possibly genetically-distinct strains of a species, some of which are able to produce the toxins,

others which apparently cannot, 3) physiological condition of the toxic cells, and 4) the effects of environmental variables on toxin production at the cellular level.

6.1 Phytoplankton dynamics - Since any method to determine the toxicity or toxin content of natural bloom material from water bodies uses samples of mixed phytoplankton, the proportion of species in the sample that are capable of producing toxins and those that cannot will greatly affect the concentration of cyanobacterial toxins present. Phytoplankton communities are highly dynamic entities, with genera and species that can boom and then crash, co-exist with or replace others on an almost continual basis throughout the year. Therefore, even if the principle producer of a toxin (*Microcystis* sp., for example) produces a toxin at a constant rate all year, changes in the abundance of that species over time (hours, days, months, years) will dramatically affect the toxicity of the phytoplankton community as a whole. For example, Carmichael and Gorham (1981) reported dramatic differences between the toxicities of phytoplankton (detected by mouse bioassay) collected from 26 sites (some only 10 m apart) in Hastings Lake, Alberta. Some of the variation was attributed to differences in the phytoplankton species composition between sites. This spatial variation in toxicity was termed a "mosaic pattern of toxicity". Lindholm and Meriluoto (1991) studied the vertical distribution of the cyanobacterium *O. agardhii* and a demethylated MC-RR analogue throughout 1989 in Lake Ostra Kyrksundet, Finland. The vertical profile of the toxin changed over the year, tracking the vertical position of *O. agardhii* in the water column. The highest toxin concentration (30 μg cellular toxin L^{-1}) was found in July in the metalimnion at a depth of 7 to 9 m, corresponding to the depth preference of *O. agardhii*. Although much of the variability in cyanobacterial toxicity data reported is often attributed to changes in phytoplankton species composition, no studies have attempted to statistically relate (e.g., by correlation analysis) toxin concentrations (or mouse bioassay toxicity) to either abundance or biomass (biovolume) of specific phytoplankton species.

6.2 Genetic control of toxin production - Very little is known about the genetic regulation of toxin production in freshwater cyanobacteria. Although changes in the proportion of genetically distinct strains has often been used to explain some of the variability in the reported toxicities of blooms over time, no study has examined this.

However, strains that do not produce the toxins do exist in nature. When separate colonies of *M. aeruginosa*, originating from a single toxin-producing phytoplankton sample, are cultured under ideal toxin-producing conditions in the laboratory, some of the isolated strains did not produce microcystins. Production could be controlled by genetic mechanisms internally (by the toxin-producing cells) or by plasmids since more than half of isolated cyanobacteria contain plasmids (Herdman 1982). Vakeria et al. (1985) examined whether plasmids could control microcystin production in cultured strains of *M. aeruginosa*. They found that cultures grown in the presence of novobiocin (a plasmid "curing agent") were just as toxic by mouse bioassay as cultures not receiving novobiocin treatment. Electrophoresis of *M. aeruginosa* DNA showed that plasmid DNA was absent in those cultures grown in novobiocin.

Although no other direct genetic work has been attempted on toxic strains of cyanobacteria, interest has been increasing steadily in the last five years in the biosynthetic pathways of cyanobacterial hepatotoxin production. Gjolme et al. (1991) reported that production of microcystins in *M. aeruginosa* was not dependent on traditional ribosomal pathways and speculated that the toxins may be formed via a protein synthase. Rinehart et al. (1994) was the first to propose the complete biosynthetic pathway for nodularin. The first step is the conversion of phenylacetic acid to a polyketide that is then methylated and converted to Adda. Adda then condenses with D-Glu and the dipeptide with N-methylthreonine, which then loses a water molecule. The compound then condenses with B-methylaspartic acid and then arginine to form linear nodularin. The last step is the cyclization of the linear peptide, also initiating its toxicity. The biosynthesis of MC-LR was suggested to be very similar to nodularin, except that D-Ala and L-Leu are inserted following the dehydroamino acid, and the dehydroamino acid is formed from serine rather than threonine (Rinehart et al. 1994).

6.3 Physiological condition of cyanobacterial cells - Several studies have indicated that microcystin production in laboratory cultures of *M. aeruginosa* is linked closely to the growth phase that the cyanobacteria are in (Van der Westhuizen and Eloff 1985; Watanabe and Oishi 1985; Ohtake et al. 1989; Kaya and Watanabe 1990). Laboratory cultures of cyanobacteria and other algae undergo four growth phases in

culture: 1) an initial lag phase, when growth is slow, 2) exponential growth, when growth rate is maximal and mortality (senescence) is low, 3) a stationary phase when growth rate equals the rate of senescence, and 4) a death phase when senescence exceeds growth (Fogg 1975). Under culture conditions, microcystin concentration in the cells increases dramatically from the lag phase and peaks in late exponential phase. However, this pattern can be altered quite dramatically by altering environmental conditions. For example, Sivonen (1990) found that [D-Asp³]MC-RR production in two laboratory strains of *O. agardhii* deviated substantially and variably from the expected growth phase relationship when water temperature or light intensity was changed. Therefore, the mosaic pattern of toxicity in a lake described by Carmichael and Gorham (1981) may also be a reflection of the differences in growth phases that the cyanobacteria are in between sites in a lake. However, it is unknown whether the growth phase of a population of a particular species in one area of a water body is synchronous with populations in other locations within the same water body. If synchronous, the change in growth phase over a summer may also explain some of the variability in toxicity within a year, if one considers the seasonal growth pattern of a species within a water body to be representative of the classical laboratory growth curve (Fogg 1975).

Since the concentration of toxin detected in phytoplankton samples is dependent on both production of the toxin and losses, loss of the toxin from the cells may also account for some of the variability. During the stationary and death phases of cyanobacteria in culture, microcystin concentration in the cells can decrease precipitously. The cause of this decrease has not been studied in detail but has been suggested to be caused by leakage of the toxins from the cells or breakdown of the toxins by the cyanobacteria. Senescing blooms or cultures in poor physiological condition have been shown to release microcystins into the surrounding water (Berg et al. 1987; Sivonen 1990; Kenefick et al. 1993). Therefore the physiological condition of the cyanobacterial cells can have a dramatic influence on the toxin concentrations in the cells by influencing both production and loss of toxins.

6.4 *Effects of environmental factors on toxin production* - Of all the types of studies undertaken relating to cyanobacterial toxins, the effects of various environmental

factors on cyanobacterial neuro and hepatotoxin production has the longest history. This is likely due to the need to culture cyanobacteria with high toxin producing capabilities. Almost all of these environmental factor studies have been performed on laboratory cultures. Extrapolation from these laboratory experiments to what may occur in natural lake settings is difficult, and likely not valid since many of the laboratory studies provide conflicting results (discussed later). No experiments have been conducted at a mesocosm scale (e.g., in lake enclosures or limnocorrals). Only one study has attempted to correlate toxin concentrations in natural phytoplankton assemblages from a reservoir to limnological conditions in the water body (Wicks and Thiel 1990). However, since this single waterbody is located on another continent (Africa) and in a very different climatic regime compared to Canada (the reservoir is not ice-covered during the winter months and water temperatures are always above 15 °C), the validity of extrapolating their results to lakes on the Canadian prairies is questionable.

Environmental factors that have been studied in laboratory experiments have included light intensity and quality (Van der Westhuizen and Eloff 1985; Watanabe and Oishi 1985; Sivonen 1990; Utkilen and Gjølme 1992; Rapala et al. 1993), phosphorus and nitrogen concentration (Watanabe and Oishi 1985; Sivonen 1990; Rapala et al. 1993), water temperature (Van der Westhuizen and Eloff 1985; Watanabe and Oishi 1985; Sivonen 1990; Rapala et al. 1993) culture pH (Van der Westhuizen et al. 1988) and trace metals (Fe, Al, Cd, Cr, Cu, Mn, Ni, and Sn; Lukac and Aegerter 1993). Unfortunately, several studies examining the same environmental factors (temperature, for example) can report differing effects on toxin production, thus making generalizations difficult. For example, Sivonen (1990) found that *O. agardhii* strain 79 grew best and produced more [D-Asp³]MC-RR at 25 °C but grew more slowly and produced less toxin at 15, 20 and 30 °C. In contrast, Watanabe and Oishi (1985) found that the growth of *M. aeruginosa* increased with increasing temperature (18 to 32 °C) and that microcystin production (estimated by mouse bioassay) was not different at the three temperatures. The differences in growth and toxin production between these two studies probably reflected differences in the two genera used (*Oscillatoria* and *Microcystis*). However, even within a species, marked differences are still observed. For example, the response of *O. agardhii*

strain 79 (described above; Sivonen 1990) to changes in temperature was substantially different from *O. agardhii* strain CYA 128, in which growth in the latter strain was equal at all four temperatures and toxin production similar at 15, 20 and 25 °C, and lower only at 30 °C.

Nutrients such as phosphorus and nitrogen have long been suspected to control toxin production by cyanobacteria. As with the above-mentioned studies, the magnitude of response, and even the direction of response to changes in phosphorus and nitrogen alterations in culture media differs between studies. For example, Sivonen (1990) found that orthophosphate concentration had a marked effect on [D-Asp³]MC-RR production in *O. agardhii* at low P concentrations (100 to 400 µg L⁻¹) and not at higher P concentrations, while Watanabe and Oishi (1985) found little change in the toxicity of *M. aeruginosa* under culture conditions when phosphorus concentration in the media was reduced to 1/10 and 1/20 of the original culture concentration. However, the original concentration of phosphorus was very high (100 mg L⁻¹ of orthophosphate), and even at 1/20 of the original concentration, phosphorus was not limiting growth appreciably. Rapala et al. (1993) examined the effect of nitrogen concentration and source (N₂, NO₃⁻, NH₄⁺ and urea) on the production of the cyanobacterial neurotoxin, anatoxin-a, in several laboratory strains of *Aphanizomenon* and *Anabaena*. They found that toxin production was highest when cells were grown in N-free media and was lowest when grown in N-rich media. In contrast, Sivonen (1990) found that production of [D-Asp³]MC-RR in *O. agardhii* increased with increasing nitrate concentration. The differences in the response of toxin production to nitrogen or phosphorus manipulation is not surprising since the response might be expected to be species-dependent. In addition, the function of anatoxin-a and microcystins in the cyanobacteria may be very different, and therefore, the response to changes in nitrogen and phosphorus would be expected to differ.

No studies have attempted to identify which types of lakes have the highest potential for microcystin-producing phytoplankton and what variables set the potential. All studies that have evaluated phytoplankton toxicity (by mouse bioassay) or actual microcystin concentrations in the phytoplankton (by HPLC) have been conducted on eutrophic to hypereutrophic lakes (although nutrient (N or P) or chlorophyll *a*

concentrations are rarely mentioned). No studies have determined if the potential for microcystin production within lakes is related to nutrient concentrations. It is commonly held that the ratio of nitrogen to phosphorus (as total nitrogen:total phosphorus) can be a key determinant of the biomass potential of cyanobacteria in lakes (Smith 1983), with little cyanobacterial biomass being present in lakes with a ratio greater than approximately 30:1, and anywhere from little biomass to massive growths of cyanobacteria at a ratio less than 30:1. The influence of the ratio of nutrients, such as nitrogen and phosphorus, on microcystin production in lakes is unknown.

Another way of examining the effect of environmental conditions on toxin production is to correlate toxin concentrations found in natural phytoplankton samples to limnological or environmental conditions in a water body over time. Wicks and Thiel (1990) provide the only study that has done this. They examined the concentration of six microcystin analogues in phytoplankton samples in Hartbeespoort Dam, South Africa over a 2.5 yr period and correlated the changes in microcystin concentration to various environmental variables. Microcystin concentrations were positively correlated to biomass-normalized primary production, solar radiation, oxygen saturation of the water, water temperature and pH and weakly negatively correlated to chlorophyll *a* and orthophosphate concentrations. The applicability of their results to a wider range of lakes (e.g., to lakes on the Canadian prairies) is doubtful since Hartbeespoort Dam is never ice-covered during the winter months, water temperature did not fall below 15 °C, and nutrient concentrations were well in excess of phytoplankton requirements (e.g., usually > 200 µg L⁻¹ of orthophosphate and > 1,000 µg L⁻¹ of nitrate). Additionally, several of the above parameters in the Wicks and Thiel (1990) study were autocorrelated and sample size was small (n=16 to 20).

Correlations between toxin concentrations in phytoplankton and environmental variables also have other limitations. For example, since toxin concentration in natural phytoplankton samples can be greatly influenced by phytoplankton species composition, one cannot determine which factors are influencing toxin concentration in the phytoplankton by affecting the abundance of the toxin-producing species, and which factors are regulating actual toxin production or loss within those species. One could

begin to tease apart the affects of environmental variables on species composition and toxin production at the cellular level if one species in the lake was responsible for the production of the toxin. Toxin concentrations could then be expressed per unit biomass of the one species and correlations between microcystin concentration (per unit biomass of *M. aeruginosa*, for example) and environmental variables, and microcystin concentration and species abundance could then be compared.

7. Toxicity of Microcystins to Animals

As mentioned earlier, much of the knowledge base for the effects of microcystins on animals can be accredited to detailed pathological reports, usually by veterinarians, after animal poisoning incidents and through initial detection of microcystins in phytoplankton samples by use of mouse bioassays. Today much of the toxicological research is still carried out on laboratory rodents (mice, rats), although numerous toxicological studies have focused on species that are commonly poisoned, such as cattle (Carmichael et al. 1977), sheep (Jackson et al. 1984; Runneger et al. 1988), pigs (Beasley et al. 1986; Falconer et al. 1994) and chickens (Jackson et al. 1987; Falconer et al. 1992b). In addition, more recent studies have begun to determine if microcystins are toxic to aquatic animals such as zooplankton (DeMott et al. 1991; Reinikainen et al. 1994) and fish (Phillips et al. 1985; Sugaya et al. 1990; Rabergh et al. 1991; Andersen et al. 1993; Beveridge et al. 1993).

7.1 LD₅₀ and the dose-response curve - It is a commonly held misconception that anthropogenic contaminants are among the most highly toxic compounds ever evaluated. This view is primarily a result of mass media coverage of isolated and accidental releases of contaminants such as PCBs (polychlorinatedbiphenyls) into the environment. However, natural toxins are far more toxic, based on mouse toxicity tests, than anthropogenic contaminants (Table 4). For example, Botulinum toxin is 2.66×10^9 times more toxic than the synthetic insecticide malathion. Cyanobacterial toxins such as saxitoxin, microcystins and the anatoxins are also much more toxic than malathion. Dioxin (specifically the 2, 3, 7, 8 congener) has been touted as the most toxic compound ever synthesized by humans, yet its toxicity pales in comparison to most natural toxins.

Cyanobacterial toxins such as MC-LR are up to 12 times more toxic than 2, 3, 7, 8-TCDD in mice. As a reference, compounds that have an LD₅₀ less than 5 mg kg⁻¹ are considered to be supertoxic (Klaassen et al. 1986).

Although LD₅₀ values in mice by intraperitoneal injection have been reported for some of the microcystins, no studies have actually shown what the dose response curves look like. The shape of the dose response curve is an important component of an LD₅₀. Two compounds may have the same LD₅₀ but differ greatly in the response of the animals to doses on either side of the LD₅₀ value. For example, for one compound, very little mortality may occur below the LD₅₀ value while doses only slightly higher than the LD₅₀ may cause almost 100% mortality in the animals, while for another compound the curve may be gentler. The dose-response curve for the former compound is said to be very steep since movement away from the LD₅₀ would mean the difference between life and death for most animals. For the latter however, movement above or below the LD₅₀ may result in only a slight change in mortality. Microcystins have a very steep dose response curve (Carmichael pers. comm.). Jackson et al. (1984) reported that the dose-response for sheep to hepatotoxic *M. aeruginosa* was very steep, where up to 90% of the lethal dose could be ingested without any measurable effects on the animal.

7.2 Routes of exposure - The route of exposure causing animal poisonings is by ingestion of cyanobacterial toxin-containing water and/or cyanobacterial cells when the animals drink from water sources containing high concentrations of toxins. This most often occurs when the cyanobacteria are producing a high concentration of the toxin per unit biomass, and when the cyanobacteria accumulate along shorelines where animals have access to water. Accumulation along shorelines can occur when wind speed is negligible, creating calm conditions on the lake and the buoyant cells float to the lake surface (Reynolds 1978). A gentle wind may then blow the cells to a windward shore. Poisonings of animals by cyanobacterial toxins occur sporadically but repeatedly worldwide each year. Animals can die in as little as a few minutes after ingestion to several days later, depending on the toxin concentration in the cyanobacteria, the amount of water and cells ingested, type of cyanobacterial toxin(s) present, type and physiological condition of the animal, and the amount of food in the stomach (Carmichael and Gorham 1977).

Interestingly, some animals appear to be highly susceptible to cyanobacterial toxins by an oral route of exposure while others are not. This may reflect differences in physiological resistance of the animals and/or the differences in the efficiency of the digestive tract to extract the toxins from the cyanobacteria and transport the toxin across the intestinal wall into the bloodstream. For example, cows, which have digestive tracts that are efficient at breaking down complex organic matrices such as cellulose would be expected to be more susceptible to cyanobacterial toxins than mice (if the two species had an equal physiological resistance to the toxin), which have a much simpler digestive system. This hypothesis could be tested by comparing the oral:intraperitoneal LD₅₀ ratio in the two species. Unfortunately, no single study has addressed this directly. For example, Carmichael et al. (1975, 1977) found that calves died when given an oral dose of neurotoxic *An. flos-aquae* (containing anatoxin-a) equal to the intraperitoneal LD_{min} dose in mice. Since the oral LD₅₀ for anatoxin-a is up to 63 times higher than the intraperitoneal LD₅₀ in mice (Stevens and Krieger 1991), the higher toxicity in the calves may be due to more efficient extraction and transport of anatoxin-a into the bloodstream relative to mice. Alternatively, calves may be more physiologically susceptible to the toxin than mice. This cannot be answered as the intraperitoneal toxicity of the bloom material to the calves was not determined by Carmichael et al. (1975, 1977). However, it is evident that oral toxicity is usually much lower than the intraperitoneal toxicity for anatoxins, microcystins and nodularin within a particular species. For example, the ratios of the oral to intraperitoneal LD₅₀ in mice and chickens for microcystins are 62 and 126, respectively (Jackson et al. 1986; Falconer et al. 1988), >200 for nodularin in mice (Kiviranta et al. 1990), >170 for *An. circinalis* (likely containing anatoxin-a) in mice (Runnegar et al. 1988) and >>170 for *An. circinalis* in sheep (Runnegar et al. 1988).

The wide discrepancy between oral and intraperitoneal toxicity of cyanobacterial toxins probably reflects the difference in the number of biological barriers needed to be crossed before the toxic action occurs. Intraperitoneal injection is a direct route of exposure where the toxin is taken up into the bloodstream directly and rapidly from the peritoneal cavity. In contrast, in an oral exposure, the toxin must cross the stomach or intestinal wall before entering the bloodstream. The intestinal wall may therefore

completely or partially restrict the passage of the toxin to the bloodstream. Thus, in the case of microcystin, the concentration reaching the liver is much higher and the time of exposure more rapid (acute) by intraperitoneal injection than by oral exposure.

Most toxicological studies involving cyanobacterial toxins have focused on intraperitoneal exposures rather than oral exposure. This is probably due to the lower amount of toxin required for intraperitoneal studies, the shorter time period needed to complete intraperitoneal studies with lethal doses and the ease of intraperitoneal injections compared to oral intubations.

Humans can also be exposed to cyanobacterial toxins by an oral route in several ways. Although humans tend to avoid swimming and drinking water from water bodies containing heavy blooms of cyanobacteria, incidents of human poisonings, suspected of being caused by ingestion of cyanobacterial toxins have occurred when people have accidentally swallowed water while swimming or boating (Dillenberg and Dehnelt 1960; Lawton and Codd 1991) or by intentional ingestion of water contaminated with microcystin-producing cyanobacteria when alternative drinking water sources were not available (Yu 1989; Carmichael 1994). Water-borne illness in humans is discussed in more detail in Section 10 of this chapter. Humans may also be exposed to cyanobacterial toxins orally by ingesting vegetables (lettuce, cabbage) that have been irrigated with water originating from ponds, lakes or reservoirs containing toxic cyanobacteria (Murray-Darling Basin Commission 1993). Microcystins can be stable for years within dried *M. aeruginosa* cells that have been washed up along a lake shore (Gary Jones, Chair, Murray-Darling Basin Commission, pers. comm.). The hazard posed by microcystins on irrigated vegetables is not known, however.

Recently, there has been interest in the use of cyanobacteria as health food. In some cases the cyanobacteria is harvested directly out of hypereutrophic lakes which support high biomasses of cyanobacteria. For example, one product (Super Blue-Green™, marketed by Cell Tech) is harvested from Klamath Lake, Oregon. The product claims that the contents of its product is 100% *Aph. flos-aquae*, although I have personally examined the product under a dissecting microscope and found other cyanobacteria (*Microcystis* spp. for example) and zooplankton. Given that the species composition of the

phytoplankton cannot be regulated in the product, it is very likely that the species composition of the product will change between harvesting dates, possibly including dates when microcystin-producing *Microcystis* spp. are present. *Microcystis* spp. has been shown to be present in surface scums along the shorelines of Klamath Lake (J. Kann, Klamath Lake Tribe, unpublished data). I analyzed seven phytoplankton samples collected from Klamath Lake from August to September 1991. All contained MC-LR. In addition, other peaks in the chromatograms may have corresponded to other microcystins, although without other microcystin standards I was not able to confirm this. *Aphanizomenon flos-aquae* has been shown to produce PSPs, toxins which have caused fatalities in humans after PSP-containing shellfish have been ingested (Subba Rao et al. 1988; Silvert and Subba Rao 1992). PSPs are five times more toxic than MC-LR (Table 1 and 2). In fact, Klamath Lake is one of the very few locations worldwide where toxic *Aph. flos-aquae* has been isolated (Gentile 1971). Given the rather high recommended dosages for this health food (1 to 2 g d⁻¹), it is conceivable that the amount of microcystin, for example, ingested would exceed a suggested tolerable daily intake (TDI) value of 13.3 ng kg body weight⁻¹ d⁻¹ (Kuiper-Goodman 1994). Therefore, ingestion of this 'health food' may result in a much higher and acute exposure to cyanobacterial toxins than drinking contaminated water.

Two other routes of exposure are possible for cyanobacterial toxicity: dermal and inhalation exposure. Wannemacher et al. (1988) applied MC-LR (dissolved in 90% DMSO) to the skin of guinea pigs at a dose of 500 µg kg⁻¹ (40 µg cm⁻²). They reported no mortalities but mild skin irritation was observed. Therefore microcystins are only slowly absorbed by the skin, even with penetrants such as DMSO. It is unlikely that microcystins are the cause of reported cases of contact dermatitis after humans swam in cyanobacterial-infested water bodies. Other compounds produced by cyanobacteria have been shown however, to cause contact dermatitis (see Carmichael et al. 1985). Creasia (1990) found that nasal uptake of MC-LR in mice was possible and that the LD₅₀ by this route of exposure was 18 µg L⁻¹ of air. If aerosols containing microcystins are formed naturally, beside a lake on windy days for example, the concentrations are not likely to cause adverse health affects.

7.3 *Acute versus chronic exposure* - Microcystins represent both an acute and chronic health risk. Acute exposures have resulted in substantive losses of livestock (>1,000 animals) over a period of days, and have also resulted in spectacularly large die-offs of wildlife and waterfowl. One of the best examples of the latter case was from Storm Lake, Iowa in the early 1950s (Rose 1953). Losses included 7,000 Franklin gulls, 1,000 ducks, 200 pheasants, 50 squirrels, 18 muskrat, 15 dogs, 4 cats, 2 hawks, and 1 each of fox, skunk and mink over one summer. Poisoning of animals is therefore not restricted to those animals that can drink large quantities of water (such as cattle). Carmichael and Gorham (1977) estimated that ingestion of as little as a few mL of water and cells could be sufficient to kill animals, if the toxicity of the bloom material was high.

Animals that frequent the same water source continually over the summer, or those that tend to water only from lakes that contain cyanobacteria may also be chronically exposed to cyanobacterial toxins. Under this scenario, small quantities of the toxins are consumed over a long period of time. This aspect of cyanobacterial toxicology is just now beginning to be explored, especially as it relates to human health. This concern has developed based on two important findings. Firstly, that microcystins (and anatoxin-a) are not removed adequately by conventional water treatment processes (Falconer et al. 1989; Himberg et al. 1989) and secondly, that microcystins and nodularins are now known to be potent tumour promoting agents. This will be discussed in more detail later. Therefore, in terms of human health effects, chronic exposure to microcystins may represent more of a health hazard than acute exposure.

7.4 *Uptake and physiological effects of microcystins in mammals* - Most of the toxicological work done on microcystins has involved either mice or rats by intraperitoneal injection. Exposure of mice by this route causes massive hemorrhage in the liver with death generally occurring in less than two hours post-injection (Falconer et al. 1981). Numerous aspects of the toxicity of microcystins have been studied, ranging from uptake kinetics from the intestine, tissue distribution, biotransformation and excretion and detailed work on the effects on hepatocytes (liver cells) at the cellular level (histology and ultrastructure) and biochemical level.

Uptake of microcystins under natural conditions would occur through absorption by the intestine after the microcystins have been ingested either in water or in toxic cyanobacterial cells. Two regions of the intestine are responsible for absorption of the toxin: the ileum and jejunum (Dahlem et al. 1989). Absorption occurred primarily through the ileal region while the jejunal region was about half as effective at transporting MC-LR (Dahlem et al. 1989). It is hypothesized that transport of the toxin across the intestine occurs via the multispecific bile acid transporter, a carrier-mediated process by which many substances are transported to the bloodstream (Dahlem et al. 1989; Falconer et al. 1992b). In addition to transporting the microcystin into the hepatic-portal blood vessels, the enterocytes (cells lining the intestine) themselves, which contain the multispecific transport mechanism, can be destroyed by the microcystin (Falconer et al. 1992b). In some cases, gastroenteritis, a condition of severe stomach cramps and diarrhea in humans, may reflect destruction of cells lining the intestine after accidental ingestion of microcystin.

Tissue distribution studies have examined the fate of microcystins in mammalian tissues. Three types of radiolabelled microcystins (tritiated and ^{14}C -labeled MC-LR and -RR, and iodinated MC-YM) have been used to study tissue distribution in mice. Tritiation appears to be the method of choice for labeling, giving both high specific activity and high label stability (Robinson et al. 1989; Meriluoto et al. 1990b). Methods of labeling with ^{14}C resulted in an analogue with low specific activity (Brooks and Codd 1987), making it difficult to perform detailed biotransformation work, and labeling based on ^{125}I resulted in an analogue with low label stability (Runnegar et al. 1986). Furthermore, an ^{125}I label can only be used with microcystin analogues that contain tyrosine (e.g., MC-YM).

Once in the bloodstream, microcystins are quickly localized into the liver. Brooks and Codd (1987) found that 70% of the administered ^{14}C -labeled MC-LR was found in the liver one min after intraperitoneal injection into mice. Similarly, Robinson et al. (1989) found that up to 60% of ^3H -labeled MC-LR accumulated in the liver very quickly (in 30 to 60 min). The high specificity of microcystins for the liver can be explained by the presence of the multispecific bile acid transporters present in the hepatocyte plasma membranes. These or similar transport systems are also found in intestinal and kidney cells (Frimmer

and Ziegler 1988; Falconer et al. 1992b). Consequently, radioactively-labeled microcystins are also localized, although to a lesser extent than in the liver, in the intestine and kidneys (Robinson et al. 1989; Meriluoto et al. 1990b). The lack of multispecific transporters also explains the lack of pathological effects of microcystins on other cell types (human erythrocytes, ovary tissue, smooth muscle, human hepatocarcinoma cell line Hep G2, mouse fibroblast cell line NIH-3T3, human neuroblastoma cell line SH-SY5Y; Eriksson et al. 1987, 1990a).

Evidence for the role of the multispecific bile acid transporters in the toxicity of microcystins was first suspected when pretreatment of mice or rats with compounds that block bile acid transporters abolished or decreased mortality significantly in animals subsequently dosed with microcystins (Stoner et al. 1990; Hermansky et al. 1991; Mereish et al. 1991). These "chemoprotectants" were then shown to offer protection by blocking the uptake of microcystins by the hepatocytes (Runnegar et al. 1981, 1991; Eriksson et al. 1990a; Mereish and Solow 1990).

Pathological effects to the liver has been studied by intraperitoneal or intravenous injection of mice and rats with purified MC-LR or cyanobacterial extracts containing the toxin (Falconer et al. 1981; Theiss et al. 1988; Hooser et al. 1989; Miura et al. 1989, 1991) and by perfusing isolated, whole rat livers with MC-LR in solution (Berg and Soli 1985b; Berg et al. 1988; Theiss et al. 1988). Lesions are easily seen by visual observation. Livers from mice that had been exposed to MC-LR by intraperitoneal injection are dark red and engorged with blood (Falconer et al. 1981; Slatkin et al. 1983; Theiss et al. 1988). Histological lesions (i.e., lesions seen by light microscopy) in the liver begin centrilobularly (i.e., originate in the center of each lobule) and progress in severity from hydropic edema (swelling) to disassociation of the hepatocytes, and in the most severe cases, hepatocyte necrosis and lysis of the plasma membranes (Berg et al. 1988; Hooser et al. 1989). Nuclear changes such as pyknosis and karyokinesis are also observed (Berg et al. 1988). Disassociation of the hepatocytes may lead to a rapid influx of blood from the rest of the body into the liver, causing the massive hemorrhage. During this time, there is a noticeable progression of pallor (loss of color) in the eyes, ears and tail of the mice, as up to 44% of the total blood volume of the body can end up pooling in the liver (Adams et al.

1988). As a result, the mass of the liver relative to the body may increase by two fold or more (Falconer et al. 1981; Brooks and Codd 1987). Occasionally, disassociated hepatocytes become trapped in the central veins (Hooser et al. 1989) where they may then travel to, and become trapped in, the lungs.

Such severe destruction of the liver can cause a significant release of enzymes, commonly found in high concentration in the liver, into the bloodstream. If the normal "operating range" of the enzymes in the plasma are known for a species, activities of the enzymes can be used as a diagnostic tool for assessing liver trauma or damage. Enzymes such as lactate dehydrogenase (LDH), alanine aminotransferase (ALT), aspartic acid aminotransferase (AST), sorbitol dehydrogenase (SDH) and gamma-glutamyl transferase (GGT) have been used widely as indices of hepatic damage in mice and rats dosed with MC-LR (Falconer et al. 1981; Berg et al. 1988; Miura et al. 1989). In addition, such enzymes have also been used to retrospectively assess human exposure to microcystins in a drinking water supply in Australia after the reservoir containing a heavy bloom of *M. aeruginosa* was treated with copper sulfate (Falconer et al. 1983a).

The pathology of MC-LR to the liver in mice has also been extensively studied at the ultrastructure level, utilizing scanning and transmission electron microscopy. Lesions caused by intraperitoneal injection of mice and rats with MC-LR progresses from swelling of the hepatocytes, to widening of the space of Disse accompanied by hepatocyte disassociation, plasma membrane invagination, vesiculation of rough endoplasmic reticulum (RER), swelling of mitochondria and loss of ribosomes from RER (Dabholkar and Carmichael 1987; Hooser et al. 1990). Large membrane-bound vacuoles that commonly form within the cells may be caused by separation or fragmentation of the ER.

Runnegar et al. (1981) were the first to report pathological effects of a unidentified peptide toxin from *M. aeruginosa* *in vitro* on isolated hepatocytes. Scanning electron microscopy showed large evaginations of the plasma membrane, forming what they termed surface blebs within 5 min of exposure. Since then others have reported similar blebs with microcystins isolated from other cyanobacteria (Eriksson et al. 1988; Hooser et al. 1991). Because of the similarity of pathological lesions caused by microcystins (massive hemorrhage in the liver and blebbing of hepatocytes) with that of the mushroom

toxin, phalloidin, Runnegar et al. (1981) hypothesized that the blebbing observed in hepatocytes treated with microcystin might be caused by interactions of the toxin with the cell cytoskeleton, as is the case for phalloidin. Runnegar and Falconer (1986) found that while both phalloidin and microcystin caused blebbing of isolated hepatocytes, only phalloidin caused a marked decrease in the ratio of G-actin to F-actin. Therefore, the mechanism of cytoskeletal change between the two toxins differed. It was not until 1989 that the mode of action of microcystins began to be elucidated. After extensive work with isolated rat hepatocytes, Eriksson et al. (1989a) concluded that the blebs observed in microcystin-treated hepatocytes was not caused by pathways reported for other toxins including alteration of intracellular thiol levels, loss of calcium homeostasis, or ATP depletion. By using a combination of electron microscopy and fluorescent staining of actin, Eriksson et al. (1989a) found that hepatocyte blebbing was associated with a rapid reorganization of microfilaments in the cells. The cytoskeletal elements were polarized to one end of the cell, causing the blebs. They concluded that microcystins were a novel microfilament perturbing toxin. Hooser et al. (1991) subsequently confirmed this by *in vitro* studies with cultured hepatocytes and *in vivo* in rats. However, these results did not provide the mode of action of the toxins. Did the microcystin bind directly to the microfilaments or was there an intermediate pathway involved?

Several independent researchers (Eriksson et al. 1990b; Honkanen et al. 1990; MacKintosh et al. 1990; Yoshizawa et al. 1990), provided the last piece of the puzzle to explaining the mode of action of microcystin when they reported that microcystins are potent inhibitors of the enzymes, protein phosphatase 1 and 2a. Protein phosphatases and protein kinases control a number of important and diverse processes in cells including muscle contraction, structure of the cytoskeleton during cell division and glycogen metabolism (Chou et al. 1989; Cohen 1992). Control of these processes is by reversible dephosphorylation and phosphorylation of serine, threonine and tyrosine amino acid residues on target proteins by protein phosphatases and protein kinases, respectively. Inhibition of protein phosphatases by microcystins then explained the commonly reported depletion of glycogen stores in the livers of rats exposed to MC-LR (Miura et al. 1991; Pace et al. 1991). The conversion of glycogen to glucose is dependent on the the

dephosphorylation of glycogen synthase by protein phosphatases and the phosphorylation of glycogen phosphorylase by protein kinases. In a normal cell, the breakdown of glycogen to glucose or the synthesis of glycogen from glucose is dependent on the balance between dephosphorylation by protein phosphatases and phosphorylation by protein kinases (Cohen 1992). The inhibition of protein phosphatases 1 and 2a also explained earlier observations of an increase in the activation of phosphorylase a in hepatocytes exposed to microcystins (Runnegar et al. 1987). It therefore was postulated that microcystins (and nodularin) exert their toxic effect on the hepatocellular cytoskeleton through the inhibition of protein phosphatases which causes hyperphosphorylation of the proteins of the cytoskeleton (Eriksson et al. 1990b; Ohta et al. 1992; Falconer and Yeung 1992).

The inhibition of protein phosphatases by microcystins, similar to that of another known protein phosphatase inhibitor and tumor promoter, okadaic acid, lead to the hypothesis that microcystins may also possess tumor-promoting capabilities. Tumor promotion occurs in cells only after the genetic material (i.e., DNA) is already damaged (i.e., after a tumour initiation step), by exposure to benzene, arsenic, radiation or alcohol for example. The promotion stage of carcinogenesis then results in the rapid proliferation of cells, producing benign tumours. The inhibition of protein phosphatases has been suggested as a mechanism of tumour promotion (Fujiki 1992). The inhibition of protein phosphatases would result in the hyperphosphorylation state and subsequent inactivation of tumour suppresser proteins. Nishiwaki-Matsushima et al. (1992) have shown that microcystins, through the inhibition of protein phosphatases, can promote tumor formation in rat livers initiated with diethylnitrosamine (DEN). In addition, microcystins appear to be one of the most potent tumor promoters known. It is interesting to note that the geometric isomers of MC-LR and RR ([6(z)Adda⁵]MC-LR and -RR), which are not toxic to mice by intraperitoneal injection, and show a low capability to inhibit protein phosphatases, are also weaker tumour promoters than their parent toxins which are highly toxic to mice and which inhibit protein phosphatases to a much greater extent (Nishiwaki-Matsushima et al. 1991).

Few studies have determined the fate of microcystins in animal tissue. Robinson et al. (1991) reported that more than 23% of a sublethal dose of tritiated MC-LR was excreted in mice over a six day period: approximately 9% in urine and 15% in feces. At 6 and 12 h post-injection, urine and feces were analyzed by HPLC for the parent toxin and metabolites. Approximately 60% of the recovered toxin was the parent toxin. The remainder consisted mainly of more polar metabolites. These metabolites may be the parent MC-LR conjugated to glutathione and cysteine. Conjugation of xenobiotics to glutathione or cysteine had been suggested as a metabolic pathway leading to detoxification (Ketterer et al. 1983). Conjugation usually results in a more polar and less toxic metabolite. However, glutathione conjugation has also been reported to increase the toxicity of certain compounds (Igwe 1986). Runnegar et al. (1987) reported that glutathione pools were significantly depleted in isolated rat hepatocytes treated with MC-YM and Hermansky et al. (1991) found that pretreatment of mice with glutathione 2 h before administration of MC-LR protected the mice completely against microcystin-caused mortality. This led Kondo et al. (1992) to suspect that glutathione or cysteine conjugation might play an important role in the detoxification of microcystins and may explain the presence of metabolites less polar than MC-LR in the urine and feces of MC-LR-exposed mice reported by Robinson et al. (1991). To determine this, Kondo et al. (1992) synthesized MC-LR, -YR and -RR conjugates of glutathione and cysteine and examined their toxicity relative to their parent toxins, and their chromatographic behavior relative to their toxic parents by HPLC. The conjugated microcystins were less toxic than their corresponding toxic parent compounds, but surprisingly, all conjugates were still highly toxic (LD_{50} from 217 to 630 $\mu\text{g kg}^{-1}$, intraperitoneal in mice). The reduced, but still significant toxicity of the conjugates was likely a result of the lack of structural change in the Adda moiety of the microcystins (Kondo et al. 1992), the component of microcystins which confers most of the toxicity (Harada et al. 1990a+b). Kondo et al. (1992) also found that the microcystin conjugates were also less hydrophobic as determined by HPLC. Therefore, the metabolites observed by Robinson et al. (1991) may have been MC-LR conjugated to glutathione or cysteine. Thus, biotransformation of microcystins by animals may be possible.

Robinson et al. (1991) and Pace et al. (1991) studied the subcellular distribution of tritiated MC-LR in the liver from mice exposed to MC-LR by intravenous injection and in perfused rat livers, respectively. Both studies found that most of the microcystin in the liver was bound to cytosolic protein within the hepatocytes. Subsequently, strong evidence has been presented that suggests that the proteins are in fact protein phosphatases (Toivola et al. 1994).

8. Toxicity of Cyanobacterial Toxins to Zooplankton

Much interest has developed recently concerning whether cyanobacterial neurotoxins and hepatotoxins can have an impact on aquatic animal communities. Part of this interest originated from the hypothesis that cyanobacterial toxins may be an evolutionary response to grazing pressure by zooplankton (Lampert 1981a). Initial support for this hypothesis came largely from field studies that noted dramatic declines in zooplankton population size and shifts in zooplankton community composition from large bodied cladocerans to smaller bodied cladocerans, copepods and rotifers when cyanobacteria dominated the phytoplankton community in water bodies (Gliwicz 1977; Edmonson and Litt 1982; Ocrutt and Pace 1984; Gilbert 1994). Several mechanisms have been proposed to explain this phenomenon. These include low nutritional quality of the cyanobacteria, low manageability and possibly toxicity. Cyanobacteria are generally of much lower nutritional quality than other groups of algae such as greens and diatoms (Arnold 1971). In addition, cyanobacteria may be more difficult to handle during feeding. Thus, manageability may limit their utilization (Gliwicz 1977). Several workers have also suggested that certain species of cyanobacteria are directly toxic to the zooplankton. If the production of toxins by cyanobacteria is a response to zooplankton grazing, one might also expect the zooplankton to respond by evolving mechanisms to reduce their susceptibility to such toxins.

In the past decade or so, numerous studies have evaluated the toxicity of neuro- and hepatotoxin-producing cyanobacterial strains and the purified toxins, to various species of zooplankton (summarized in Table 5). A toxic effect is concluded to occur when the zooplankton (in the presence of other nutritionally adequate species such as

Chlamydomonas sp. and *Scenedesmus* sp.) die more quickly when a toxic strain is added than when the zooplankton are starved. For example, Lampert (1981b) reported 100% mortality of *Daphnia pulicaria* within 4 d when held in containers containing a food base consisting of 50% or more *M. aeruginosa* and 50% or less *Scenedesmus* sp. In contrast, less than 60% mortality of *D. pulicaria* occurred over 6 d when the zooplankton were starved. The mechanisms and indeed the compounds responsible for mortality in zooplankton are still the subject of debate. Mortality, or lack thereof, in zooplankton exposed to toxic cultures of cyanobacteria is probably related to a number of factors, and very possibly I suspect, not to cyanobacterial toxins at all.

One of the most commonly reported effects of toxic cyanobacteria on cladocerans is an immediate reduction in filtering rate (see Table 5). This is commonly examined by radiolabelling a food source (e.g., *Scenedesmus* sp.) and measuring the ingestion rate of the alga in the presence of toxic and non-toxic cyanobacteria. While filtering and ingestion rates are usually much lower in the presence of cyanobacteria than in the presence of green algae or diatoms (Arnold 1971; Fulton 1988a; DeMott and Moxter 1991), ingestion rates can be much lower for toxic forms of cyanobacteria than for their non-toxic counterparts that are morphologically identical (DeMott and Moxter 1991; DeMott et al. 1991). This line of evidence can be used to support that view that the reduction in feeding rate is a defense mechanism by the zooplankton to avoid ingesting toxic cells. This assumes that the zooplankton can discriminate between toxic and non-toxic cyanobacteria based on chemical cues, cues that are presumably present within the cyanobacterial cells. One would expect that zooplankton species that avoid ingesting toxic cyanobacteria cells, or ingest them minimally, would suffer less mortality than species which appear not to be inhibited at all by toxic forms, providing that the species have an equal physiological resistance to the toxin. For example, DeMott et al. (1991) reported a relatively good survivorship in *Daphnia pulicaria* that was associated with a rapid feeding inhibition in the presence of toxic *M. aeruginosa* cells and a poor survivorship of *D. pulex* that was attributed to uninhibited feeding on the toxic *M. aeruginosa*. This supported the hypothesis that feeding inhibition is an adaptive strategy by the zooplankton.

The poor survivorship of cladocerans such as *D. pulex*, reported by DeMott et al. (1991) when filter feeding activity was uninhibited by the presence of a toxic strain of *M. aeruginosa* suggests that compounds in the cells may be toxic to the zooplankton. In those studies that have concluded that a certain strain of cyanobacteria was toxic to a zooplankter, the toxic agent responsible for the mortality was often assumed to be one of the toxins (microcystins, anatoxins) that cause mortality in mammals, usually without any substantive evidence. However, DeMott et al. (1991) did determine the toxicity of purified MC-LR and nodularin in solution to several cladocerans and a copepod. *D. pulicaria* was the least sensitive species to MC-LR (48 h LC_{50} of $21.4 \mu\text{g mL}^{-1}$), followed by *D. hyalina* ($11.6 \mu\text{g mL}^{-1}$) and *D. pulex* ($9.6 \mu\text{g mL}^{-1}$). *Diaptomus bergei* was the most sensitive (0.45 to $1.0 \mu\text{g mL}^{-1}$). Therefore there can be marked differences in the physiological susceptibilities to MC-LR between zooplankton species. DeMott et al. (1991) and DeMott and Moxter (1991) concluded that the coexistence of zooplankton with toxic forms of cyanobacteria would depend on both the physiological resistance of the zooplankton to the toxins, and also the behavioral responses in feeding. Thus, the good survivorship in *D. pulicaria* when placed in a culture of toxic *M. aeruginosa* was due to low sensitivity to the toxin and rapid filter feeding inhibition, and low survivorship of *D. pulex* was due to greater physiological sensitivity to the toxin and uninhibited feeding (DeMott et al. 1991). Even though *D. bergei* was the most sensitive to MC-LR, good survivorship was due to active discrimination against ingesting toxic cells (DeMott and Moxter 1991; DeMott et al. 1991). Therefore, while cladocerans may have adapted to the presence of toxic cyanobacteria by avoiding ingesting toxic cells (by reducing filtering rates), copepods may reduce the chance of ingesting toxic cells by feeding preferentially on non-toxic forms of the same species, or on other species of cyanobacteria.

Physiological resistance to cyanobacterial toxins has also been hypothesized to be an evolved response to the toxins (DeMott et al. 1991). One would therefore expect that zooplankton species that usually coexist with toxic cyanobacteria would be more resistant to the toxins than species that are never exposed to such toxins. In support of this hypothesis, Gilbert (1990) found that *D. pulex* coexisting in a lake with toxic *Anabaena*

affinis was resistant to its toxic effects, whereas *D. pulex* isolated from another source, presumably lacking toxic *An. affinis* (although this was not stated), was much more sensitive. Similarly, Gilbert (1994) stated that the low sensitivity of the rotifer *Brachionus calyciflorus* to anatoxin-a was likely an evolutionary response to its greater tendency to ingest toxic *Anabaena flos-aquae* filaments relative to other rotifers studied.

Despite all of the evidence mentioned in this section that suggests that microcystins and anatoxins within the cyanobacterial cells can cause selective mortality of some zooplankton species, and hence can possibly alter the species composition of zooplankton communities, recent studies have challenged this view. For example, while DeMott et al. (1991) found that purified aqueous MC-LR was toxic to several species of cladocerans and to a copepod, the reported LC_{50} values (0.45 to $21.4 \mu\text{g mL}^{-1}$ or 450 to $21,400 \mu\text{g L}^{-1}$) are 500 to almost $25,000$ times higher than reported dissolved concentrations of all microcystin analogues combined in natural settings (i.e., in hypereutrophic Alberta lakes; see Lambert et al. 1994a). Given that just about any compound can be considered toxic if in high enough concentration, it is unlikely that environmentally realistic concentrations of MC-LR (generally $< 1 \mu\text{g L}^{-1}$) would be toxic to zooplankton. In addition, Reinikainen et al. (1994) determined the LC_{50} of toxic *M. aeruginosa* cells to *D. pulex* to be $30,000$ cells mL^{-1} , corresponding to $0.001 \mu\text{g}$ of cellular MC-LR mL^{-1} . In contrast DeMott et al. (1991) reported an LC_{50} of $9.6 \mu\text{g mL}^{-1}$ for dissolved MC-LR in *D. pulex*. The difference in LC_{50} spans almost four orders of magnitude. Since dissolved MC-LR would have to cross a number of external barriers (e.g., the respiratory surface) to get into the zooplankton whereas MC-LR in *M. aeruginosa* cells would enter the zooplankton by ingestion, the much higher LC_{50} of the dissolved toxin may reflect lower penetrability of the toxin through external membranes (respiratory surfaces) compared to the gut. Alternatively, the toxicity of dissolved MC-LR reported by DeMott et al. (1991) may be an artifact of extremely high concentration, and therefore the toxicity of *M. aeruginosa* cells and extracts to zooplankton could be caused by other compounds in the cells, unrelated to microcystins.

Jungmann et al. (1991) were the first to address the issue relating to the possibility that compounds in *M. aeruginosa* other than microcystins may be responsible for the

toxicity observed in zooplankton. Jungmann et al. (1991) found that cells of *M. aeruginosa* strain PCC 7806 were highly toxic to mice and *Daphnia* sp. However, after the cells were extracted with water, the cells themselves (not the extract) were still toxic to mice, but filter feeding inhibition in *Daphnia* sp. was reduced and there was no mortality in *Daphnia* sp. When the cells were then extracted in methanol, the cells were less toxic to mice, but did not inhibit filter feeding activity by *Daphnia* sp, nor did it cause any mortality in *Daphnia* sp. They then concluded that *Daphnia* toxicity and inhibition of filtering rate was caused by different compounds, and that mouse toxicity and filter feeding inhibition were caused by the same compound(s). Based on their experimental design and their results I would submit an alternate hypothesis. I would contend that there was no evidence to suggest that different compounds in the extract were responsible for toxicity in *Daphnia* sp., and filtering inhibition. I believe that their results can be explained by differences in the efficiency of toxin removal from the cells by the two solvents. Since the level of toxicity of the cells to mice was natural cells > water-extracted cells > methanol-extracted cells, the loss in toxicity to *Daphnia* sp. after the natural cells were extracted with water probably was due to just enough removal of the toxic compound(s) by the water extraction to abolish *Daphnia* sp. toxicity but not enough loss to abolish filtering inhibition (which would be expected to still occur at much lower concentrations than what would cause death). When the cells were then extracted in methanol, a solvent with much higher extraction efficiency than water, the toxicity of the cells to the mice dropped dramatically. During this extraction, most if not all of the toxic constituents were removed from the cells. Therefore, no filter feeding inhibition was observed in the *Daphnia* sp. Therefore, the differences in the effects of the cell extraction steps (changes in toxicity and filter feeding inhibition in *Daphnia* sp.) only reflect differences in extraction efficiencies of the solvents used, and does not suggest the existence of separate compounds which 1) kill mice and *Daphnia* sp, and 2) cause filter feeding inhibition. From this study, the toxic and inhibitory actions of microcystins on *Daphnia* sp. cannot be dismissed, yet.

Subsequently however, Jungmann (1992) did provide evidence that suggested that microcystins may not be responsible for the toxicity of *M. aeruginosa* to zooplankton. In

this study Jungmann extracted *M. aeruginosa* cells and partially purified the extract with disposable C-18 cartridges (those used commonly as a preparative step in sample cleanup for HPLC). The crude extract contained 22 $\mu\text{g mL}^{-1}$ of MC-LR and 12.3 $\mu\text{g mL}^{-1}$ [D-Asp³]MC-LR. The LC₅₀ of the crude extract to *D. pulicaria* was 47 $\mu\text{l mL}^{-1}$ (3.1 $\mu\text{g mL}^{-1}$ of the two toxins). The crude extract, containing the microcystins was then passed through a C-18 cartridge. The cartridge-passed extract contained no detectable microcystin (by HPLC) yet caused 100% mortality in *D. pulicaria*. In contrast, the extract fraction that was subsequently eluted from the C-18 cartridge in methanol, containing the two microcystins in similar concentration to the original crude extract, caused almost no mortality in the *D. pulicaria*. This provided the first strong evidence that compounds other than microcystins, found in cyanobacteria, may be responsible for the observed toxicity to zooplankton. In a later study, Jungmann and Bendorf (1994) found no correlation between total microcystin concentration (detected by HPLC) in the extracts of laboratory strains of *M. aeruginosa* and natural phytoplankton, and *D. pulicaria* toxicity (48 h LC₅₀). Thus, Jungmann and Bendorf (1994) concluded that the compound(s) responsible for toxicity in zooplankton are not related to microcystins. They termed the unknown compound *Daphnia*-toxic compound (DTC).

The mechanism(s) of toxicity to zooplankton of microcystins, anatoxins and DTC are not known. For microcystins, given their high specificity for tissues containing membrane-bound multispecific transporters, (cells in the liver, kidney and intestinal tract) one would expect pathological effects on similar cell types in zooplankton. I am not aware of any reports that have determined whether zooplankton contain an organ analogous to the mammalian liver (such as the heptaopancreas in shellfish). Behavioral observations of zooplankton immersed in dissolved MC-LR (DeMott et al. 1991), including loss of swimming ability and unresponsiveness, give no clues as to the mechanism of toxicity. Similarly, the mechanism of toxicity of anatoxin-a to rotifers (Gilbert 1994) is not known. If the toxin operates similarly in zooplankton as it does in mammals, one would expect to see jerky and frenetic swimming movements in the exposed zooplankton, caused by the neurotoxic effects on nerve synapses. Unfortunately, no behavioral details were described in the paper (Gilbert 1994).

9. Accumulation of Cyanobacterial Toxins in Aquatic Invertebrates

Few studies have evaluated whether cyanobacterial toxins are transferred to aquatic invertebrates which feed directly on the toxin-containing cyanobacteria, and if toxins can then be transferred to other organisms that feed on toxin-contaminated invertebrates. Watanabe et al. (1992) examined the concentration of three microcystin analogues (MC-LR, -RR and -YR) in a zooplankton community in hypereutrophic Lake Kasumigaura, Japan. The zooplankton community contained high concentrations (75-1387 $\mu\text{g g}^{-1}$) of the three microcystins. Although this accumulation was hypothesized to be mainly a result of the filter feeding activities of one dominant zooplankter, *Bosmina fatalis*, no attempts were made to correlate microcystin concentrations in the zooplankton community to zooplankton species composition. It is presently unknown whether the microcystins were accumulated in the zooplankton tissue or whether the toxin was contained within the *Microcystis* sp. cells within the gut of the zooplankton. The current dogma in the literature would suggest that water-soluble compounds (such as microcystins) may not accumulate in animal tissues, particularly to the same extent as dioxins in mammals.

Eriksson et al. (1989b) examined the concentration of a microcystin analogue (detected by HPLC) in the freshwater mussel, *Anodonta cygnea*, exposed to a hepatotoxic culture of the cyanobacterium *Oscillatoria agardhii*. The mussels contained high concentrations of the microcystin in the tissue (70 $\mu\text{g g}^{-1}$ after a 15 d exposure). However, the mussels were also exposed to high concentrations of the microcystin in pure culture (2000-3000 $\mu\text{g toxin g}^{-1}$ of *O. agardhii* or 40-60 μg of cellular toxin L^{-1}). Eriksson et al. (1989b) also examined the concentration of the toxin in different organs of the mussels. They found the highest concentration of the microcystin in the hepatopancreas (>120 $\mu\text{g g}^{-1}$), followed by the intestine and gonads. Little microcystin was detected in the muscle.

Lastly, nodularin has been detected in blue mussels (*Mytilus edulis*) collected during a hepatotoxic bloom the cyanobacterium *Nodularia spumigena* in Peel-Harvey estuary, Western Australia (Falconer et al. 1992a). Gut contents of the mussels contained

filaments of *Nodularia spumigena* and the highest toxicity (detected by mouse bioassay) was associated with the gastrointestinal tract of the mussels. No toxicity was observed for non-intestinal tissues, although nodularins may have been present but below the detection limit by mouse bioassay.

It is not known whether other aquatic invertebrates that feed on different trophic levels in aquatic food webs (herbivores such as gastropods and other zooplankton species, invertebrate predators such as some cyclopoid copepods and macroinvertebrates, detritivores such as *Gammarus* sp.) can also accumulate microcystins by their feeding activities. It is probable, based on the three above studies, that invertebrate predators (micro and macroinvertebrates, fish, muskrat, birds) could be exposed to such toxins by ingesting toxin-containing invertebrate food stuffs. This needs further attention.

9. Toxicity of Microcystins to Fish

Although long suspected as a cause or contributing factor to massive fish kills, the link between the occurrence of cyanobacterial toxins and sudden die-offs of large numbers of fish during the summer months in eutrophic and hypereutrophic lakes has yet to be made. Many of these "summerkill" events are associated with periods of hot, calm weather. Warm water temperatures coupled with a lack of turbulent diffusion of oxygen into the water column under these conditions decrease the oxygen-holding capacity of the water (Wetzel 1983). In addition, such climatic conditions also favor the formation and development of cyanobacterial blooms. Sudden mortalities of fish during these heavy blooms are thought to be caused by hypoxic stress or toxicity to ammonia as a result of the collapse and subsequent decomposition of the algal bloom (Barica 1975). However, there have also been incidents when fish mortalities have occurred after the collapse of cyanobacterial blooms when dissolved oxygen in the water was well above the requirement for survival of the fish species affected (Schwimmer and Schwimmer 1968; Penaloza et al. 1990). A sudden release of toxins from the cyanobacterial bloom has been postulated to be partly or solely responsible for the observed fish mortality, although this has been unsubstantiated. Uptake of cyanobacterial toxins from the water, via the gills,

has only been examined once. Unfortunately, this experiment also had some flaws making any conclusion difficult.

Phillips et al. (1985) exposed rainbow trout (*Oncorhynchus mykiss*) to a *M. aeruginosa* (Strain PCC 7820) culture in 30 L aquaria. This strain was shown to be highly toxic to mice and the fish by intraperitoneal injection. Biomass of *M. aeruginosa* in the aquaria was very high at the beginning of the experiment ($> 200 \mu\text{g L}^{-1}$ of chlorophyll *a*) and decreased to $< 50 \mu\text{g L}^{-1}$ over the course of the 10-d exposure. No fish mortality was observed and no histological lesions in the liver of the fish were evident after the 10 d. Phillips et al. (1985) contend that since chlorophyll *a* concentration decreased markedly over the exposure period, the fish should have been exposed to microcystins that would be expected to be released from the degrading cells. However, microcystin concentration in the water was not determined. Further, I would argue that given the very high pigment concentrations in the water, the microcystin may have degraded or isomerized to non-toxic isomers (e.g., [6(z)Adda⁵]microcystins) as found by Tjusi et al. (1994). Therefore the effects of exposure of fish to dissolved cyanobacterial toxins, such as microcystins, remains unresolved.

The above findings however, do not imply that microcystins are not toxic to fish such as rainbow trout. Indeed, Phillips et al. (1985) found that microcystin was acutely toxic to rainbow trout by intraperitoneal injection, as did Rabergh et al. (1991) studying effects on carp (*Cyprinus carpio*), Sugaya et al. (1990) studying goldfish (*Carassius auratus*) and Andersen et al. (1993) studying Atlantic salmon (*Salmo salar*). As with mammals, intraperitoneal injection of microcystin-containing extracts of *M. aeruginosa* cells or purified toxins caused severe degeneration of the liver in the above fish species. Histological lesions of the liver included hepatocellular swelling, disassociation of hepatocytes, and severe hepatic necrosis (Phillips et al. 1985; Sugaya et al. 1990; Rabergh et al. 1991; Andersen et al. 1993). Electron microscopy of the liver from carp exposed to MC-LR by intraperitoneal injection showed swollen mitochondria, degranulation of RER (i.e., loss of ribosomes) and hepatocyte disassociation (Rabergh et al. 1991), similar to the ultrastructural lesions observed in mice and rats that were injected intraperitoneally with MC-LR (Dabholkar and Carmichael 1987; Miura et al. 1989). Also in common with

studies examining the pathological effects on mice and rats, was an increase in the activities of the enzymes LDH, ALT and AST in the plasma of carp exposed to MC-LR, an indication of severe hepatic stress (Rabergh et al. 1991).

In contrast to mice and rats, Rabergh et al. (1991) also reported that histological lesions in the kidney were evident in carp injected intraperitoneally with MC-LR. These lesions included dilation of Bowman's space and nuclear pyknosis in some tubule cells. These lesions however, were not nearly as severe as those observed in the liver of the fish, and have rarely been reported in mice or rats.

Fish species appear to be more tolerant of high microcystin doses and also survive longer than mice and rats. For example, the lowest consistently lethal dose of intraperitoneally-administered MC-LR to carp was $550 \mu\text{g kg}^{-1}$, a dose 11 times higher than the LD_{50} of MC-LR in mice ($50 \mu\text{g kg}^{-1}$; Carmichael 1988). Sugaya et al. (1990) determined the 24 h LD_{50} of MC-RR in goldfish to be 2.6 mg kg^{-1} , three to 10 times higher than in mice (Table 2). In addition, Andersen et al. (1993) injected Atlantic salmon intraperitoneally three times (each injection was three days apart) at a dose of $555 \mu\text{g kg}^{-1}$ injection⁻¹. Only one of the 25 fish died, and one more was moribund over the 36 d experiment. In addition to the apparent lower toxicity of MC-LR in fish compared to mice, the fish also can survive a lethal dose much longer than mice. While mice will die in less than two h after intraperitoneal injection of a lethal MC-LR dose (Carmichael 1988), Andersen et al. (1993) reported survival up to 23 d in Atlantic salmon, and Sugaya et al. (1990) reported survival of goldfish up to 3 d after injected with a lethal dose of MC-RR. I believe that the longer survival and higher tolerance of fish to MC-LR than mice may lie in the rate at which MC-LR is taken up by the liver. Gingerich (1982) states that the perfusion rate of blood through the liver in fish such as rainbow trout can be one-quarter that of mammals. Therefore, the exposure of the fish liver to intraperitoneally-administered MC-LR might be much more chronic in nature than in mammals. While the mouse liver is exposed to the toxin in a rapid pulse, the fish liver might be exposed to the toxin over a long period of time. This hypothesis might also explain the occurrence of kidney lesions in fish exposed to MC-LR but not in mice, and the presence of regenerating "islands" of hepatocytes in a milieu of necrotic hepatocytes, as reported by Andersen et

al. (1993) in salmon. If the perfusion of the fish liver is much lower than in mammals, the toxin may remain in circulation longer, thus having a chance to reach the kidney in higher concentration and thus causing kidney lesions in the fish. Also, longer survival of the fish would also increase the likelihood of the production of regenerative hepatocytes in the fish liver, as reported by Andersen et al. (1993). Alternatively, the longer survival and higher dose tolerance to microcystins shown by fish may also be due to slower uptake kinetics of the toxins by the fish hepatocytes. This has not received any attention. Runnegar et al. (1991) found that the uptake of MC-YM by isolated rat hepatocytes was dramatically decreased by decreasing ambient temperature. Since most fish exist in an environment that is much colder than the mammalian core body temperature (37 °C), a slower uptake of microcystins by fish hepatocytes based only on ambient temperature could explain the longer survival and higher dose tolerance of fish compared to mice. In addition, fish hepatocytes may also have different uptake kinetics than mammalian hepatocytes at equivalent temperatures. Again, this has not been studied.

Exposure of fish to microcystins by intraperitoneal injection however, is not a natural route of exposure. Aside from uptake of the toxins via the gills, the only other plausible route of exposure to fish may be ingestion of either the toxic cells directly or ingestion of food items that contain the toxin as a result of filter feeding activity (e.g., zooplankton [Watanabe et al. 1992], mussels [Eriksson et al. 1989a; Falconer et al. 1992a]). Beveridge et al. (1993) found that tilapia and silver carp, which feed directly on phytoplankton, had significantly lower opercular beat rates when exposed to microcystin-containing *M. aeruginosa* cells versus non-toxic *M. aeruginosa* cells. Therefore a lower volume of water and food particles was passed over the gills in the fish exposed to toxic cells. Certain species may therefore be able to avoid ingesting toxic cells through modification in feeding behavior.

Sugaya et al. (1990) are the only investigators that have examined the oral toxicity of a microcystin to fish. They mixed freeze-dried and disrupted *M. viridis* cells (containing MC-RR) with the fishes' regular pellet food and allowed the fish to feed on the contaminated pellets for 28 d. No mortality occurred during the 28-d exposure, and only mild pathology to the liver was observed in three out of 20 fish (liver enlargement,

and slight loss of color). The cause of the liver enlargement (e.g., hemorrhage) was not determined. By extrapolation from their data, the cells contained about 4.7 mg of MC-RR g^{-1} of cells. Since the pellets contained 1% *M. viridis* cells by weight and the fish ingested about 10 g $\text{kg}^{-1} \text{d}^{-1}$, the dose of MC-RR that the fish were exposed to would have likely been only 0.047 mg $\text{kg}^{-1} \text{d}^{-1}$. Therefore the lack of significant pathological effects are not surprising since this may be considered a very low dose by oral exposure. More work needs to be done relating to the oral toxicity of microcystins to fish, particularly with respect to dose response of the liver at the histological and cell ultrastructure level. Additionally, it is important to link the concentrations of microcystins in aquatic invertebrates (which may be a food source for certain species of fish) to the potential for adverse effects on fish populations. Such an assessment of risk has not been made for fish.

One series of studies has provided strong evidence that microcystins can have a severe impact on commercially-important fish stocks in the environment. Rearing of Atlantic salmon in netpens along the coast of British Columbia, Canada and the State of Washington, U.S.A. is a multi-million dollar industry. Since 1986 salmon reared in netpens have died in large numbers and displayed severe liver lesions, termed netpen liver disease (NLD; Kent et al. 1988; Kent 1990). The liver lesions observed in salmon from the netpens (Kent et al. 1988; Kent 1990) were identical to those caused by intraperitoneal exposure of the salmon to MC-LR in laboratory experiments (Andersen et al. 1993). Through experimentation, NLD in fish held in netpens was not related to diet, could not be experimentally transmitted (therefore was not due to viral or bacterial action), was not related to anthropogenic contamination and progressed in severity over the summer in each year. This led them to suspect a natural toxin. Subsequently, Andersen et al. (1993) successfully isolated a compound from the livers of salmon inflicted with NLD and from biota fouling the netpens (utilizing HPLC-linked protein phosphatase assay) that was chromatographically-indistinguishable from MC-LR. MC-LR was not detected in the livers of fish not afflicted with NLD. Not only was this the first study to document a direct (and severe) effect of a cyanobacterial toxin on fish populations in the field, but also demonstrated for the first time that microcystins are produced in the marine environment. The organism responsible for the production of the MC-LR however, has not yet been

reported. It was suggested that the fish were exposed to the MC-LR by feeding on the organisms fouling the netpens. These organisms were also shown to contain MC-LR (Andersen et al. 1993). Given these results it would appear that microcystins may have an effect on natural fish populations in freshwater lakes, and, at times, the impact may be substantial (e.g., death). More research needs to be directed toward the impact of such toxins on freshwater biota.

10. Human Health Concerns Due to Exposure to Water-borne Cyanobacterial Toxins

There have been numerous reports of human illness suspected of being caused by exposure to cyanobacterial toxins in water bodies or in drinking water. Most of the cases are based on circumstantial evidence, many in retrospect. Although there have been cases that have resulted in severe illness in humans, no human fatalities have been linked to exposure to cyanobacterial toxins. In contrast, fatalities have been reported after humans have consumed tainted shellfish containing the paralytic shellfish poisons, saxitoxin and neosaxitoxin. Table 6 summarizes some of the reported cases of human health effects suspected of being caused by cyanobacterial toxins. The most common symptoms in humans after accidental contact with or ingestion of water suspected to contain such toxins include skin rashes and blistering, fever, nausea, vomiting and severe diarrhea (gastroenteritis) and hepatoenteritis. For example, in September of 1989, eight soldiers reported having influenza-like symptoms after performing 360° canoe rolls and swimming exercises in Rudyard Lake, U.K., which contained a heavy bloom of *M. aeruginosa* (Lawton and Codd 1991). A few days to two weeks prior to this incident, 25 sheep and 15 dogs died as a result of swimming in Rutland Water, located in a nearby region, which contained *M. aeruginosa* that was producing MC-LR. The symptoms of the soldiers occurred one day after contact with the water and included sore throat, blistering around the mouth, abdominal pain, and vomiting and diarrhea. Two soldiers were hospitalized. One of the soldiers had elevated activities of liver enzymes in the blood, indicative of microcystin poisoning. Cases of gastro- and hepatoenteritis in humans after exposure to *Microcystis* spp., may be explained by the effects of microcystins on enterocytes lining the intestine (Falconer et al. 1992b) and on hepatocytes (Runnegar et al. 1981), respectively.

In another extreme case of human poisoning, a retrospective analysis showed a marked increase in liver enzyme activities (e.g., GGT) in the blood coincident with the chemical treatment (copper sulfate) of a heavy bloom of *M. aeruginosa* in a drinking water reservoir (Malpas Dam) in Australia (Falconer et al. 1983a). A marked increase in the enzyme GGT, indicative of hepatic injury, occurred in a population obtaining their drinking water from this source while a nearby population receiving their water from another source did not show an increase. The increase in GGT was not related to other possible factors such as an outbreak of infectious disease, hepatitis or an alcoholic binge. Falconer et al. (1983a) surmised that the microcystins released into the water from the *Microcystis* spp. cells as a result of copper sulfate treatment, passed through the water treatment system and appeared in the general distribution system. All of the cases in Table 6 are assumed to be due to acute exposure to cyanobacterial toxins.

Recently, more attention has focused on the potential for adverse effects of chronic exposure to low concentrations of cyanobacterial toxins, such as microcystins, in humans. This is probably a direct result of the discovery that microcystins are capable of inhibiting protein phosphatases at very low concentrations (MacKintosh et al. 1990) and hence are also capable of promoting liver tumours (Fujiki and Suganuma 1993). The increased attention is also a result of the observation that conventional types of drinking water treatment (including flocculation, filtration and chlorination) are not very effective at removing dissolved microcystins (described previously). Thus a potential for chronic exposure of humans to microcystins may exist. There also may exist conditions whereby humans may be exposed to concentrations of microcystins in their drinking water that are much higher than what may be expected to be present in water that has been treated. For example, in certain areas of Quidong County (Jiangsu Province, China) pond water or rainwater that is collected in ditches is used as sources of drinking water. Many of these ditches have also been reported to contain excessive growths of cyanobacteria (Nishiwaki-Matsushima et al. 1992) that produce microcystins (W. Carmichael, pers. comm. in Fujiki and Suganuma 1993). In these areas, the incidence of primary liver cancer can be up to 12 times higher (72.3 cases per 100,000 for ditch water, 141.4 cases per 100,000 for pond

water) than in nearby areas where drinking water is obtained from wells or rivers (11.7 cases per 100,000; Yu 1989).

To make a valid assessment of the risk that microcystins in drinking water may pose to humans, both epidemiological evidence and experimental toxicity research is used. Assessing the potential effects of microcystins on humans is difficult because few epidemiological studies are available. Most of the toxicological work with microcystins have been done in mice, by intraperitoneal injection. Only two studies have involved repeated oral doses in mice (Falconer et al. 1988; Kiviranta et al. 1990) and only one was carried out over a long period of time (Falconer study: up to one yr). In addition, others have questioned the validity of using mice as an animal model to assess human health effects. Falconer et al. (1994) studied the oral toxicity of a microcystin (the analogue was not specified) to pigs. They argued that pigs represent a much better animal model for extrapolation to humans because of the similarities in the gastrointestinal tract, liver and kidney function, body weight and metabolic rate between humans and pigs.

To derive a guidance value for a "safe" concentration of microcystin in drinking water, one must know either the No Observable Adverse Effect Level (NOAEL) or the Lowest Observable Adverse Effect Level (LOAEL) in the study animal (Kuiper-Goodman et al. 1994). This value is then multiplied by several uncertainty factors (to account for the mouse or pig to human extrapolation, within human variation in susceptibility, less than lifetime study, and evidence for tumor promotion) to give a tolerable daily intake (TDI) value (Kuiper-Goodman et al. 1994). A guidance value is then calculated based on the average weight of a human adult, the volume of water consumed daily and the fraction of the TDI allowed for drinking water. Kuiper-Goodman et al. (1994) derived a guidance value of $0.5 \mu\text{g L}^{-1}$ for microcystins based on mouse toxicity studies. With slightly different uncertainty factors, Falconer et al. (1994) came to a slightly higher value ($1.0 \mu\text{g L}^{-1}$).

11. Conclusions

Cyanobacteria commonly dominate the phytoplankton communities of eutrophic and hyperutrophic lakes during the summer and autumn months, and under certain

conditions, can form massive scums along shorelines. Several genera of cyanobacteria produce neurotoxic and hepatotoxic compounds which have been responsible for sporadic, but repeated poisonings of animals worldwide each year. The occurrence of toxin-producing cyanobacteria is likely much more widespread than is commonly reported based on animal poisonings and surveys based on detection of the toxins by mouse bioassay, although this remains largely an untested hypothesis. Microcystins, a family of more than 50 cyclic hepatotoxins produced by several genera of cyanobacteria, are remarkably stable to hydrolytic and enzymatic degradation, as well as to bacterial degradation. Although several studies have evaluated the effects of various environmental factors on toxin production by cyanobacteria, the validity of extrapolating such results to natural lake environments is questionable. Few correlations between toxin concentrations in natural phytoplankton communities and environmental variables have been made, and their applicability to lakes in Canada is doubtful. Microcystins can cause irreversible and massive liver damage in mammals. Few detailed studies have evaluated the effects on fish and whether such toxins can accumulate in organisms associated with aquatic and terrestrial food webs. Only the most sophisticated and expensive water treatment procedures (activated carbon filtration and/or ozonation) are effective at removing microcystins from water (or destroying them). Conventional water treatment practices (flocculation, filtration and chlorination) are much less effective. Exposure of humans to microcystins can occur through a number of routes including accidental consumption while swimming in contaminated water bodies, ingestion of "blue-green algal health foods" that may contain the toxins, or chronic exposure to low concentrations of microcystins in treated drinking water. Microcystins are potent inhibitors of protein phosphatases 1 and 2a, and hence are able to promote tumor formation. The direct link between long-term exposure of humans to microcystins in drinking water and the incidence of primary liver cancer is now being evaluated.

Table 1. Characteristics of neurotoxins produced by freshwater cyanobacteria.

Compound	Class of toxin	Molecular weight	Mode of action	Toxicity ^{*1}
Saxitoxin	Alkaloid (Paralytic Shellfish Poison)	299	Sodium channel blockade	10
Neosaxitoxin	Alkaloid (Paralytic Shellfish Poison)	315	Sodium channel blockade	10
Anatoxin-a	Alkaloid	165	Neuromuscular stimulant	250
Anatoxin-a(s)	Organophosphate	252	Acetylcholinesterase inhibition	50

^{*1} intraperitoneal LD₅₀ in mice (μg kg⁻¹)

References

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|--------------------------------|-------------------|------------------------|
| Devlin et al. 1977 | Ikawa et al. 1982 | Carmichael et al. 1985 |
| Mahmood and Carmichael 1986a+b | | Carmichael 1992 |

Table 2. Structural variations of the cyanobacterial hepatotoxins, microcystins and nodularins.

Hepatotoxin analogue	Structure	Molecular weight	Toxicity ^{a1}
Microcystin-AR	cyclo (-D-Ala-L-Ala-D-MeAsp-L-Arg-Adda-D-Glu-Mdha-)	952	250
Microcystin-FR	cyclo (-D-Ala-L-Phe-D-MeAsp-L-Arg-Adda-D-Glu-Mdha-)	1028	250
Microcystin-HilR	cyclo (-D-Ala-L-Hil-D-MeAsp-L-Arg-Adda-D-Glu-Mdha-)	1058	100
Microcystin-HtyR	cyclo (-D-Ala-L-Hty-D-MeAsp-L-Arg-Adda-D-Glu-Mdha-)	909	80-300
Microcystin-LA	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Ala-Adda-D-Glu-Mdha-)	923	50-70
Microcystin-LAba	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Abz-Adda-D-Glu-Mdha-)		
Microcystin-LF	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Phe-Adda-D-Glu-Mdha-)	951	
Microcystin-LL	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Leu-Adda-D-Glu-Mdha-)		
Microcystin-LM	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Met-Adda-D-Glu-Mdha-)	994	50
Microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-Adda-D-Glu-Mdha-)		
Microcystin-LV	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Val-Adda-D-Glu-Mdha-)		
Microcystin-LY	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Tyr-Adda-D-Glu-Mdha-)	1001	90
Microcystin-M(O)R	cyclo (-D-Ala-L-Met(O)-D-MeAsp-L-Arg-Adda-D-Glu-Mdha-)	1028	700-800
Microcystin-RR	cyclo (-D-Ala-L-Arg-D-MeAsp-L-Arg-Adda-D-Glu-Mdha-)	1037	250-800
Microcystin-WR	cyclo (-D-Ala-L-Try-D-MeAsp-L-Arg-Adda-D-Glu-Mdha-)	1067	100-200
Microcystin-YA	cyclo (-D-Ala-L-Tyr-D-MeAsp-L-Ala-Adda-D-Glu-Mdha-)	959	60-70
Microcystin-YM	cyclo (-D-Ala-L-Tyr-D-MeAsp-L-Met-Adda-D-Glu-Mdha-)	1018	60-70
Microcystin-YM(O)	cyclo (-D-Ala-L-Tyr-D-MeAsp-L-Met(O)-Adda-D-Glu-Mdha-)	1035	56
Microcystin-YR	cyclo (-D-Ala-L-Tyr-D-MeAsp-L-Arg-Adda-D-Glu-Mdha-)	1044	70-100
Microcystin-(H ₄)YR	cyclo (-D-Ala-L-(H ₄)Tyr-D-MeAsp-L-Arg-Adda-D-Glu-Mdha-)		
[D-Asp ³]microcystin-HtyR	cyclo (-D-Ala-L-Hty-D-Asp-L-Arg-Adda-D-Glu-Mdha-)	1044	
[D-Asp ³]microcystin-LR	cyclo (-D-Ala-L-Leu-D-Asp-L-Arg-Adda-D-Glu-Mdha-)	980	50-300
[D-Asp ³]microcystin-RR	cyclo (-D-Ala-L-Arg-D-Asp-L-Arg-Adda-D-Glu-Mdha-)	1023	250
[D-Asp ³]microcystin-YR	cyclo (-D-Ala-L-Tyr-D-Asp-L-Arg-Adda-D-Glu-Mdha-)	1030	
[DMAdda ³]microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-DMAdda-D-Glu-Mdha-)	980	90-100
[ADMAdda ³]microcystin-LHar	cyclo (-D-Ala-L-Leu-d-MeAsp-L-Har-ADMAdda-D-Glu-Mdha-)	1036	60
[ADMAdda ³]microcystin-LR	cyclo (D-Ala-L-Leu-D-MeAsp-L-Arg-ADMAdda-D-Glu-Mdha-)	1022	50-60
[6(z)-Adda ³]microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-6(z)Adda-D-Glu-Mdha-)	994	>1,200
[6(z)-Adda ³]microcystin-RR	cyclo (-D-Ala-L-Arg-D-MeAsp-L-Arg-6(z)Adda-D-Glu-Mdha-)	1037	>1,200

Table 2. (continued)

Hepatotoxin analogue	Structure	Molecular weight	Toxicity ^{a1}
[D-Glu(OCH ₃) ⁶]microcystin-LR	cyclo (-D-Ser-L-Leu-D-MeAsp-L-Arg-Adda-D-Glu(OCH ₃)-MdhA-)	1008	>1,000
[D-Glu(OCH ₃ H-) ⁶]microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-Adda-D-Glu(OCH ₃ H-O)-MdhA-)	1052	>1,000
[Dha ⁷]microcystin-FR	cyclo (-D-Ala-L-Phe-D-MeAsp-L-Arg-Adda-D-Glu-Dha-)	1028	
[Dha ⁷]microcystin-HphR	cyclo (-D-Ala-L-Hph-D-MeAsp-L-Arg-Adda-D-glu-Dha-)	1028	
[Dha ⁷]microcystin-HtyR	cyclo (-D-Ala-L-Hty-D-MeAsp-L-Arg-Adda-D-Glu-Dha-)	1044	
[Dha ⁷]microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-Adda-D-Glu-Dha-)	980	250
[Dha ⁷]microcystin-RR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-Adda-D-Glu-Dha-)	1023	180
[D-Dha ⁷]microcystin-YR	cyclo (-D-Ala-L-Arg-D-MeAsp-L-Arg-Adda-D-Glu-Dha-)	1030	
[Mdhb ⁷]microcystin-LR	cyclo (-D-Ala-L-Tyr-D-MeAsp-L-Arg-Adda-D-Glu-Dha-)	1008	
[L-MeSer ⁷]microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-Adda-D-Glu-Mdhb-)	1012	150
[L-MeLan ⁷]microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-Adda-D-Glu-MeSer-)		1,000
[L-Ser ⁷]microcystin-HtyR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-Adda-D-Glu-L-Ser-)	1062	
[L-Ser ⁷]microcystin-LR	cyclo (-D-Ala-L-Hty-D-MeAsp-L-Arg-Adda-D-Glu-L-Ser-)	998	
[L-Ser ⁷]microcystin-RR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-Adda-D-Glu-L-Ser-)	1041	
[D-Asp ³ , D-Glu(OCH ₃) ⁶]microcystin-LR	cyclo (-D-Ala-L-Arg-D-MeAsp-L-Arg-Adda-D-Glu-L-Ser-)	994	
[D-Asp ³ , Dha ⁷]microcystin-HtyR	cyclo (-D-Ser-L-Leu-D-Asp-L-Arg-Adda-D-Glu(OCH ₃)-MdhA-)	1030	
[D-Asp ³ , Dha ⁷]microcystin-LR	cyclo (-D-Ala-L-Hty-D-MeAsp-L-Arg-Adda-D-Glu-Dha-)	966	200-250
[D-Asp ³ , Dha ⁷]microcystin-RR	cyclo (-D-Ala-L-Leu-D-Asp-L-Arg-Adda-D-Glu-Dha-)	1009	
[D-Asp ³ , MeSer ⁷]microcystin-RR	cyclo (-D-Ala-L-Arg-D-Asp-L-Arg-Adda-D-Glu-Dha-)		
[D-Asp ³ , L-Ser ⁷]microcystin-XR	cyclo (-D-Ala-L-Arg-D-Asp-L-Arg-Adda-D-Glu-MeSer-)	998	
[D-Ser ¹ , ADMAdda ⁵]microcystin-LR	cyclo (-D-Ala-UNK-D-Asp-L-Arg-Adda-D-Glu-L-Ser-)	1038	
[D-Asp ³ , ADMAdda ⁵]microcystin-LR	cyclo (-D-Ser-L-Leu-D-MeAsp-L-Arg-ADMAdda-D-Glu-MdhA-)	1008	160
[D-Asp ³ , ADMAdda ⁵]microcystin-LHar	cyclo (D-Ala-L-Leu-D-Asp-L-Arg-ADMAdda-D-Glu-MdhA-)	1022	
[ADMAdda ⁵ , MeSer ⁷]microcystin-LR	cyclo (-D-Ala-L-Leu-D-MeAsp-L-Arg-ADMAdda-D-Glu-MeSer-)	1040	

Table 2. (continued)

Hepatotoxin analogue	Structure	Molecular weight	Toxicity ^{*1}
Nodularin	cyclo (-D-Me Asp-L-Arg-Adda-D-Glu-Mdhb-)	824	50-60
[D-Asp ¹]nodularin	cyclo (-D-Me Asp-L-Arg-DMAdda-D-Glu-Mdhb-)	810	75
[L-Val ²]nodularin	cyclo (-D-Me Asp-L-Val-Adda-D-Glu-Mdhb-)		
[DMAdda ³]nodularin	cyclo (-D-Me Asp-L-Arg-DMAdda-D-Glu-Mdhb-)	810	150
[6(z)Adda ⁴]nodularin	cyclo (-D-Me Asp-L-Arg-6(z)Adda-D-Glu-Mdhb-)	824	>2,000
[D-Glu(OCH ₃) ⁵]nodularin	cyclo (-D-Me Asp-L-Arg-Adda-D-Glu(OCH ₃)-Mdhb-)		>1,200
[D-Me Asp(OCH ₃) ⁶], D-Glu(OCH ₃) ⁷]nodularin	cyclo (-D-Me Asp(OCH ₃)-L-Arg-DMAdda-D-Glu(OCH ₃)-Mdhb-)		>1,000

^{*1} intraperitoneal LD₅₀ in mice (μg kg⁻¹)

References:

- Botes et al. 1984
 deSilva et al. (1992)
 Krishnamurthy et al. 1989
 Rinehart et al. 1994
- Carmichael 1992; Carmichael et al. 1988a
 Harada et al. 1990a+b; 1991a+b
 Luukkainen et al. 1994
 Sivonen et al. 1990a; 1992a+b
- Craig et al. 1993
 Kiviranta et al. 1992
 Namikoshi et al. 1990; 1992 a, b, c, d
 Stotts et al. 1993

Table 3. Published reports of animals poisonings in Canada suspected of being caused by ingestion of toxic cyanobacteria.

Year	Location	Animal(s)	Suspected organism	Suspected toxin	Reference
1917-18	Fort Saskatchewan, AB.	Hogs	Blue-green algae	Not reported	Gillam 1925; Schwimmer and Schwimmer 1968
1917-18	Loughheed, AB.	Hogs	Blue-green algae	Likely a neurotoxin	Gillam 1925; Schwimmer and Schwimmer 1968
1917-18	Lake Saskatoon, AB.	Horses, cows, hogs, poultry, wild birds	Blue-green algae	Likely a neurotoxin	Gillam 1925; Schwimmer and Schwimmer 1968
1924	Fraser Lake, ON.	20 cattle	<i>Anabaena</i> sp.	Not reported	Howard and Berry 1933; Schwimmer and Schwimmer 1968
1938	Finncastle Lake, AB.	Waterfowl, shorebirds	Algae	Not reported	McLeod and Bondar 1952
1938-40, 1949	Netley Marsh, MB.	Waterfowl	Algae	Not reported	McLeod and Bondar 1952
1945	Lake Dauphin, MB.	Horses, calves, 2 pigs, cat	Algae	Likely a neurotoxin	McLeod and Bondar 1952
1948-49	Sturgeon Lake, ON.	16 cows	<i>Microcystis incerta</i> , <i>M. flos-aquae</i> , <i>M. aeruginosa</i> , <i>Anabaena</i> spp., <i>An. tortulosa</i>	Neuro and/or hepatotoxin	MacKinnon 1950; Stewart et al. 1950
1949-52	Whitewater Lake, MB.	>8,500 waterfowl	<i>Aph. flos-aquae</i> , <i>M. aeruginosa</i>	Cyanobacterial toxin and/or avian botulism	Bossenmaier et al. 1954

Table 3. (continued)

Year	Location	Animal(s)	Suspected organism	Suspected toxin	Reference
1950	Duck Lake, AB.	2 cows	Blue-green algae	Not reported	O'Donoghue and Wilton 1951
1950	Baptiste Lake, AB.	2 horses, 6 pigs, 30 chickens, 7 turkeys, 7 geese, 3 dogs, gulls, ducks, blue finch	Blue-green algae	Likely a neurotoxin	O'Donoghue and Wilton 1951
1951	Lake Dauphin, MB.	1 horse, 9 dogs	<i>Aph. flos-aquae</i> , <i>An. flos-aquae</i> , <i>M. aeruginosa</i>	Likely a neurotoxin	McLeod and Bondar 1952
1954	Hoodoo Lake, SK.	pigs	<i>An. flos-aquae</i>	Not reported	Hammer 1968
1959	Echo Lake, SK.	Dogs, geese, fish	<i>Microcystis</i> sp., <i>Anabaena</i> sp., <i>Coelosphaerium</i> sp.	Likely a hepatotoxin	Dillenberg and Dehnel 1960
1959	Kindersley Lake, SK.	2 horses	Algae	Not reported	Dillenberg and Dehnel 1960
1959	Souris River Dam, Weyburn, SK.	3 cows	<i>Anabaena</i> sp., <i>Aphanizomenon</i> sp.	Not reported	Dillenberg and Dehnel 1960; Senior 1960
1959	Lost Mountain Lake, SK.	4 dogs	Algae	Not reported	Dillenberg and Dehnel 1960
1959	Balgonie, SK.	1 dog	<i>Aph. flos-aquae</i>	Likely a neurotoxin	Dillenberg and Dehnel 1960
1959	Govan, Long Lake, SK.	2 cows	<i>Microcystis</i> sp., <i>Anabaena</i> sp.	Not reported	Dillenberg and Dehnel 1960
1959	Buffalo Pound Lake, SK.	3 cows, 6 dogs	<i>Microcystis</i> sp., <i>Anabaena</i> sp.	Not reported	Dillenberg and Dehnel 1960

Table 3. (continued)

Year	Location	Animal(s)	Suspected organism	Suspected toxin	Reference
1959	Johnson Lake, SK.	>3,000 ducks	<i>Microcystis</i> sp.	Not reported	Dillenberg and Dehnelt 1960; Gorham 1960
1959	Unnamed lake, SK.	Approx. 30 dogs	<i>Anabaena</i> sp. <i>M. aeruginosa</i> , <i>M. flos-aquae</i> , <i>An. flos-aquae</i>	Likely a hepatotoxin	Senior 1960
1959	Lacombe, AB.	14 cows	Blue-green algae	Likely a hepatotoxin	MacDonald 1960
1961	Burton Lake, SK.	2 cows	<i>An. flos-aquae</i>	Likely anatoxin-a	Gorham et al. 1964
1960-70	Buffalo Pound Lake, SK.	>20 dogs, calves	<i>An. flos-aquae</i> , <i>Aph. flos-aquae</i>	Likely anatoxin-a(s)	Carmichael and Gorham 1978
1972	Disney Lake, AB.	3 calves	<i>An. flos-aquae</i>	Likely anatoxin-a	Carmichael and Gorham 1978
1972	Beaverhill Lake, AB.	12 cattle	<i>An. flos-aquae</i>	Likely a microcystin	Carmichael and Gorham 1978
1975	Bendig's Pond, SK.	34 cattle	<i>M. aeruginosa</i> , <i>An. flos-aquae</i>	Likely anatoxin-a(s)	Carmichael and Gorham 1978
1985	Steele Lake, AB.	>1,000 bats, mallard ducks, American Wigeons	<i>An. flos-aquae</i>	anatoxin-a	Pybus et al. 1986
1993	Rochester, AB.	1 cow	<i>M. aeruginosa</i>	Likely a microcystin	Kotak, unpublished

Table 4. Comparative toxicities of natural toxins and anthropogenic compounds. LD₅₀ values given as $\mu\text{g kg}^{-1}$ body weight by intraperitoneal injection in mice.

Toxin	Source	Common name	LD ₅₀
Botulinum toxin	<i>Clostridium botulinum</i>	Bacterium	0.00003
Tetanus toxin	<i>Clostridium tetani</i>	Bacterium	0.0001
Kokoi toxin	<i>Phyllobates bicolor</i>	Poison arrow frog	2.7
Tetrodotoxin	<i>Sphaeroides rubripes</i>	Puffer fish	8
Saxitoxin	<i>Aphanizomenon flos-aquae</i>	Cyanobacterium	10
Cobra toxin	<i>Naja naja</i>	Cobra	20
Microcystin-LR	<i>Microcystis aeruginosa</i>	Cyanobacterium	50
Anatoxin-a	<i>Anabaena flos-aquae</i>	Cyanobacterium	200
Curare	<i>Chondodendron tomentosum</i>	Brazilian poison arrow plant	500
2,3,7,8-TCDD		Dioxin	100-600
Strychnine	<i>Strychnos nux-vomica</i>	Plant	500
Phallatoxin	<i>Amanita phalloides</i>	Fungus	1,800
Sodium cyanide			10,000
Malathion		Synthetic insecticide	80,000

References

- Ware 1978
National Rivers Authority 1990
Carmichael 1992

Table 5. Summary of studies of the toxicity of cyanobacteria and cyanobacterial toxins to various zooplankton.

Toxic agent used *1	Zooplankton tested	Reported effects/conclusions	Reference
<i>Microcystis aeruginosa</i> cells (toxin content of cells was not determined)	Cladocera: <i>Daphnia pulex</i>	Increasing mortality and decreasing filtering rates with increasing proportion of <i>M. aeruginosa</i> in the culture.	Lampert 1981b
<i>M. aeruginosa</i> cells (toxin content of cells was not determined)	Cladocera: <i>Daphnia pulex</i>	Decreased ingestion, assimilation, survival and reproduction relative to feeding on other cyanobacteria and green algae.	Arnold 1971
<i>M. aeruginosa</i> cells (toxin content of cells was not determined)	Cladocera: <i>Bosmina longirostris</i> , <i>Ceriodaphnia reticulata</i> , <i>Daphnia ambigua</i> , <i>D. cucullata</i> , <i>D. curvirostris</i> , <i>D. galeata</i> , <i>D. hyalina</i> , <i>D. longispina</i> , <i>D. magna</i> , <i>D. obtusa</i> , <i>D. pulex</i> , <i>D. pulicaria</i> , <i>Diaphanisoma brachyrum</i>	Marked reduction in filter feeding in <i>D. pulex</i> , <i>D. ambigua</i> , <i>D. obtusa</i> and <i>D. magna</i> , smallest reduction in filtering rate in <i>D. pulicaria</i> , <i>D. galeata</i> , <i>C. reticulata</i> and <i>B. longirostris</i> . Inhibitor also depended on total food concentration and filtering rate recovered immediately when zooplankton were placed into cultures of <i>Scenedesmus</i> sp.	Lampert 1982
<i>M. aeruginosa</i> cells and filtrates of culture media from <i>M. aeruginosa</i> (toxin content of cells or filtrate was not determined)	Rotifer: <i>Brachionus calyciflorus</i>	Reduced reproduction and cohort survivorship in containers with either toxic cells or filtrates of culture media used to grow the <i>M. aeruginosa</i> in.	Starkweather and Kellar 1987

Table 5. (continued)

Toxic agent used ^{*1}	Zooplankton tested	Reported effects/conclusions	Reference
Toxic and non-toxic <i>M. aeruginosa</i> cells (strain was identified as toxic by mouse bioassay)	Cladocera: <i>D. magna</i>	Reduced survival and filtering rate by some strains of <i>M. aeruginosa</i> , not by others. Reduction in ingestion and survival was not related to the toxicity of the cells (assessed by mouse bioassay).	Nizan et al. 1986
Toxic and non-toxic <i>Anabeana flos-aquae</i> (toxin content of the cells was not determined)	Cladocera: <i>B. longirostris</i> , <i>D. brachyurum</i> , <i>D. pulex</i> , <i>D. parvula</i> , <i>Monia micrura</i> Copepods: <i>Diaptomus reighardi</i> , <i>Eurytemora affinis</i> Rotifers: <i>B. calyciflorus</i>	Higher filtering rates and selectivity for the diatom <i>Melosira</i> sp. than for comparably sized filaments of cyanobacteria. Toxic <i>An. flos-aquae</i> filaments had a stronger inhibitory effect on zooplankton than other filamentous species, except for <i>B. calyciflorus</i> and <i>B. longirostris</i> . Selectivity of zooplankton for filamentous forms was independent of herbivore body size.	Fulton 1988a
<i>M. aeruginosa</i> cells (toxicity of strain was assessed by mouse bioassay)	Cladocera: <i>D. pulicaria</i>	Extraction of cells with different solvents changed the survival and filtering rates of <i>D. pulicaria</i> . They concluded that the toxic factors responsible for mouse toxicity and inhibition of filtering rate were identical, but toxicity to <i>Daphnia</i> was by some other unknown factor.	Jungmann et al. 1991

Table 5. (continued)

Toxic agent used ^{*1}	Zooplankton tested	Reported effects/conclusions	Reference
<i>M. aeruginosa</i> cells (toxin content of the cells was not determined)	Rotifer: <i>Brachionus rubens</i>	<i>B. rubens</i> died faster when fed <i>M. aeruginosa</i> than when starved. Filaments of non-toxic <i>An. flos-aquae</i> were ingested at a moderate rate.	Rothhaupt 1991
<i>M. aeruginosa</i> cells (toxin content of the cells was not determined)	Cladocera: <i>B. longirostris</i> , <i>Ceriodaphnia quadrangula</i> , <i>D. ambigua</i> , <i>D. brachyurum</i> , <i>Sida crystalina</i> , <i>Simocephalus serratulus</i> Rotifers: <i>B. calyciflorus</i> , <i>Keratella mixta</i> , <i>Platytas potulus</i> Copepods: <i>E. affinis</i> , <i>D. reighardi</i> , <i>Tropocyclops prasinus</i>	<i>B. calyciflorus</i> actively filtered, grew and reproduced on toxic <i>M. aeruginosa</i> cells. Toxicity to zooplankton was negatively related to inhibition of filtering. Copepods avoided ingesting <i>M. aeruginosa</i> cells while cladocerans and rotifers filtered <i>M. aeruginosa</i> cells at a similar rate as that for <i>Chlamydomonas</i> sp.	Fulton and Paerl 1987
<i>Anabaena affinis</i> filaments (toxin content of the cells was not determined)	Rotifers: <i>Asplanchna brightwelli</i> , <i>Keratella cochlearis</i> , <i>K. crassa</i> , <i>K. testudo</i> , <i>Synchaeta pectinata</i> , Cladocerans: <i>B. longirostris</i> , <i>Ceriodaphnia dubia</i> , <i>D. ambigua</i> , <i>D. galeata mendotae</i> , <i>D. magna</i> , <i>D. pulex</i>	Population growth rates of all rotifers were not affected by high filament concentrations of <i>An. affinis</i> , while growth rate of all cladocerans was affected by intermediate or low <i>An. affinis</i> concentrations. Rotifers did not feed on the <i>An. affinis</i> cells while cladocerans did. Susceptibility was related to adult cladoceran body length. Extracts of <i>An. affinis</i> were just as inhibitory as whole cells to the cladocerans, ruling out interference.	Gilbert 1990

Table 5. (continued)

Toxic agent used ^{*1}	Zooplankton tested	Reported effects/conclusions	Reference
Toxic strains of <i>M. aeruginosa</i> and <i>An. flos-aquae</i> (toxin content of the cells was not determined)	Cladocerans: <i>B. longirostris</i> , <i>D. parvula</i> , <i>D. pulex</i> , <i>M. micrura</i>	Survivorship for <i>D. parvula</i> , <i>D. pulex</i> and <i>M. micrura</i> was lower in the presence of <i>M. aeruginosa</i> or <i>An. flos-aquae</i> than when starved. In contrast, survivorship of <i>B. longirostris</i> was higher in cultures of <i>M. aeruginosa</i> or <i>An. flos-aquae</i> compared to starved controls. The mechanism of resistance to the toxins was concluded to occur post-ingestion.	Fulton 1988b
Toxic and non-toxic strains of <i>M. aeruginosa</i> (toxic strains were shown to be toxic by mouse bioassay)	Cladocerans: <i>Bosmina coregoni</i> , <i>D. galeata</i> , <i>D. longispina</i> , <i>D. magna</i> Copepods: <i>Cyclops</i> sp.	Ingestion of toxic <i>M. aeruginosa</i> was low compared to <i>Ankistrodesmus falcatus</i> or non-toxic <i>M. aeruginosa</i> . Reduction in the ingestion of <i>A. falcatus</i> when <i>M. aeruginosa</i> was added was not due to reduced filtering rate but by increased rejection of food items.	Henning et al. 1991
Cell-free filtrate of <i>An. flos-aquae</i> (toxin concentration in filtrate was not determined)	Cladocera: <i>D. pulex</i>	Reduction in survivorship, filtering rate and reproduction of <i>D. pulex</i> in cell-free filtrates of <i>An. flos-aquae</i> cultures.	Ostrofsky et al. 1983
Toxic <i>An. flos-aquae</i> (toxicity of the strain was assessed by mouse bioassay)	Rotifer: <i>Asplanchna girodi</i>	Reduced reproductive rate of <i>A. girodi</i> with increasing concentration of <i>An. flos-aquae</i> cells.	Snell 1980

Table 5. (continued)

Toxic agent used ^{a1}	Zooplankton tested	Reported effects/conclusions	Reference
Fractionated extracts of toxic <i>M. aeruginosa</i> (microcystin content of extracts was determined by HPLC)	Cladocera: <i>D. pulicaria</i>	While <i>M. aeruginosa</i> cells and a crude extract of the cells (containing microcystin-LR and [D-Asp ³]microcystin-LR) were toxic to <i>D. pulicaria</i> , a C-18 cartridge-retained fraction (containing the above two toxins) was not toxic to <i>D. pulicaria</i> . However, the C-18 cartridge-passed fraction (not containing the two above toxins) was highly toxic to <i>D. pulicaria</i> .	Jungmann 1992
Toxic <i>An. flos-aquae</i> filaments and purified anatoxin-a (toxin content of the cells was not determined. However, this strain has been previously been shown to produce anatoxin-a)	Rotifers: <i>A. girodi</i> , <i>B. calyciflorus</i> , <i>K. cochlearis</i> , <i>S. pectinata</i>	Both reproduction and survivorship were reduced in all four rotifers in the presence of <i>An. flos-aquae</i> or dissolved anatoxin-a. In contrast, filtrates of dense <i>An. flos-aquae</i> cultures did not inhibit growth in the rotifers. Sensitivity of each species was dependent on the effects of the toxin within the cells on filtering rate and the physiological resistance to the toxin.	Gilbert 1994

Table 5. (continued)

Toxic agent used ¹	Zooplankton tested	Reported effects/conclusions	Reference
Extracts of natural blooms and laboratory strains of <i>M. aeruginosa</i> (microcystin content in the extracts was determined by HPLC)	Cladocera: <i>D. pulicaria</i>	No correlation between <i>D. pulicaria</i> toxicity and total microcystin content of extracts by HPLC. Toxicity was concluded to be independent of microcystins.	Jungmann and Bendorf 1994
Toxic <i>M. aeruginosa</i> (microcystin content of the cells was determined by HPLC)	Cladocera: <i>D. pulex</i>	Increasing concentrations of <i>M. aeruginosa</i> (containing microcystin-LR) reduced the survival of <i>D. pulex</i> , but the reduction in survival was also dependent on the concentration of an alternate food source (<i>Scenedesmus</i> sp.)	Reinikainen et al. 1994
Toxic and non-toxic strains of <i>M. aeruginosa</i> , <i>An. flos-aquae</i> and various species of <i>Oscillatoria</i> . (toxic strains were shown to be toxic to mice, or contain toxins by HPLC or immunoassay detection. Toxin content of the cells in the study however was not determined)	Copepods: <i>Diacyclops bicuspidatus thomasi</i> , <i>Diaptomus bergei</i> , <i>D. oregonensis</i> , Cladocerans: <i>D. pulicaria</i>	Copepods had high clearance rates of non-toxic filaments and colonies but morphologically similar strains shown to contain potentially toxic compounds where discriminated against.	DeMott and Moxter 1991

Table 5. (continued)

Toxic agent used ^{*1}	Zooplankton tested	Reported effects/conclusions	Reference
Toxic <i>M. aeruginosa</i> , microcystin-LR, nodularin (toxic strain has been shown to produce microcystin-LR. However, the toxin content of the cells was not determined)	Cladocerans: <i>D. hyalina</i> , <i>D. pulex</i> , <i>D. pulicaria</i> Copepods: <i>D. bergei</i>	Physiological sensitivity of the zooplankton to microcystin-LR and nodularin was species dependent with <i>D. bergei</i> being most sensitive and <i>D. pulicaria</i> being the most resistant. Survivorship of the zooplankton in cultures of toxic <i>M. aeruginosa</i> depended on the physiological sensitivity of the zooplankton and behavioral modifications in feeding.	DeMott et al. 1991

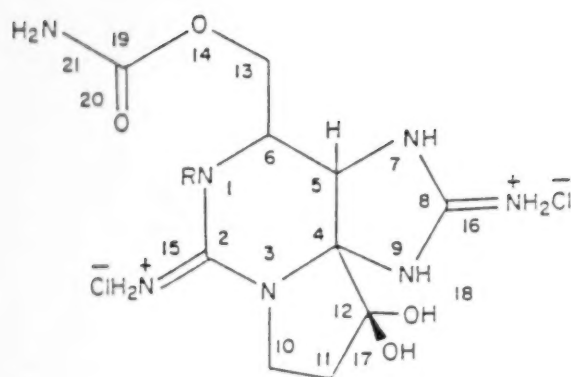
^{*1} In studies where microcystin or anatoxin concentrations in the cells were not quantified, these compounds were assumed to be responsible for any observed toxicity in the zooplankton.

Table 6. Acute poisonings of humans believed to be caused by exposure to cyanobacterial toxins.

Date and location	Circumstances and symptoms	Suspected causative agent	Reference
1887 to present, outback of northern and central Australia	vomiting, diarrhea, fever, gastroenteritis, skin rashes. "Barcoo fever"	Microcystins	Hayman 1992
1959, Saskatchewan	12 children became sick with diarrhea and vomiting after swimming in a lake containing a heavy growth of <i>Anabaena</i> sp.	<i>Anabaena</i> sp. (found in a stool sample from one of the children)	Dillenberg and Dehnel 1960
1959, Saskatchewan	One man became ill with headache, nausea and gastrointestinal upset after swimming in Katepwa Lake, SK.	<i>Microcystis</i> sp. (found in a stool sample)	Dillenberg and Dehnel 1960
1959, Saskatchewan	A physician slipped off a diving board and swallowed a half-pint of lake water. Symptoms included headache, fever, nausea, vomiting and diarrhea.	<i>Microcystis</i> sp. and <i>Anabaena</i> sp. (found in a stool sample)	Dillenberg and Dehnel 1960
1960 to 1965, Salisbury, Rhodesia	Fever, vomiting and diarrhoea in children drinking water from a source contaminated with <i>Microcystis</i> sp. The outbreaks coincided with the natural senescence of the <i>Microcystis</i> sp. blooms.	<i>Microcystis</i> sp.	Zillberg 1966

Table 6. (continued)

Date and location	Circumstances and symptoms	Suspected causative agent	Reference
1979, Palm Island, Australian coast	Outbreak of hepatoenteritis of approximately 150 people (mostly children) consuming water after a cyanobacterial bloom in a drinking water reservoir was treated with copper sulfate.	<i>Cylindrospermopsis raciborskii</i> (caused massive liver damage in mouse bioassays)	Byth 1980; Bourke et al. 1983; Hawkins et al. 1985
1981, Pennsylvania	Gastrointestinal upset, vomiting, diarrhea in 12 children and one adult after swimming.	<i>Anabaena</i> sp.	Billings 1981
1981, Armidale, Australia	Elevation of plasma enzymes GGT and ALT, indicative of hepatic injury, after a bloom of <i>Microcystis</i> sp. in a drinking water reservoir was treated with copper sulfate. A nearby population obtaining water from another source was not affected.	<i>Microcystis</i> sp.	Falconer et al. 1983a
1989, United Kingdom	Eight soldiers (two of which were hospitalized) reported sore throats, blistering around the mouth, nausea, vomiting, diarrhea after performing swimming and canoeing exercises in Rudyard Lake. One soldier had elevated activities of liver enzymes in his blood.	Microcystin-LR in <i>M. aeruginosa</i> bloom.	Lawton and Codd 1991



R = H; saxitoxin dihydrochloride
 R = OH; neosaxitoxin dihydrochloride

Figure 1. Chemical structures of the paralytic shellfish poisons, saxitoxin and neosaxitoxin. Both are neurotoxins and are produced by the freshwater cyanobacteria, *Aphanizomenon flos-aquae* and *Anabaena circinalis* (from Carmichael 1992).

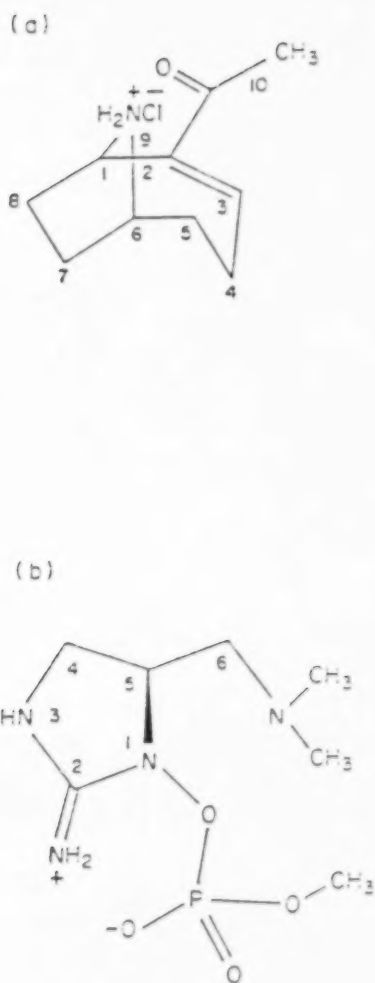


Figure 2. Chemical structures of the neurotoxins a) anatoxin-a, an alkaloid, and b) anatoxin-a(s), an organophosphate (from Carmichael 1992). These toxins are produced by species of *Anabaena*, *Aphanizomenon*, *Nostoc* and *Oscillatoria*.

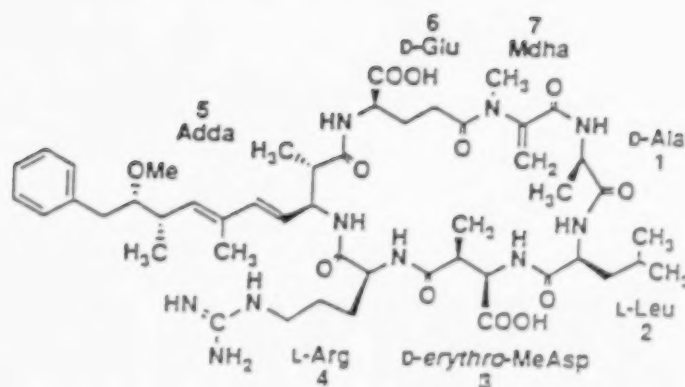


Figure 3. Chemical structure of the hepatotoxic heptapeptide, microcystin-LR, the most common and toxic microcystin produced by freshwater cyanobacteria (from Rinehart et al. 1994). This toxin is produced by species and strains of *Microcystis*, *Anabaena*, *Oscillatoria* and *Nostoc*.

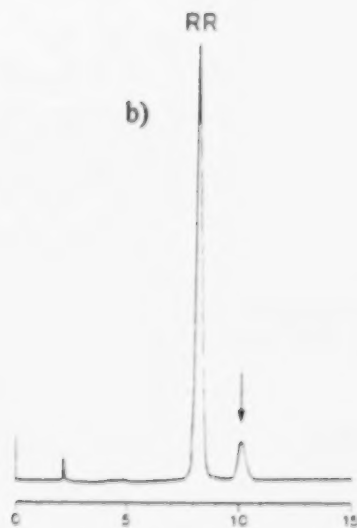
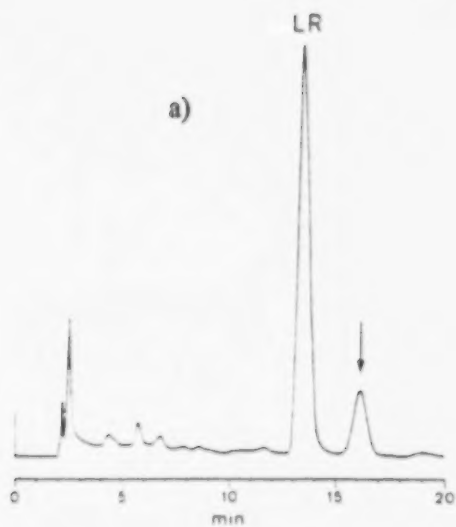


Figure 4. HPLC chromatograms showing non-toxic microcystins (indicated by arrows) associated with a) microcystin-LR and b) microcystin-RR (from Harada et al. 1990a). These peaks were subsequently identified as 6(z)Adda⁵microcystins-LR and -RR, respectively.

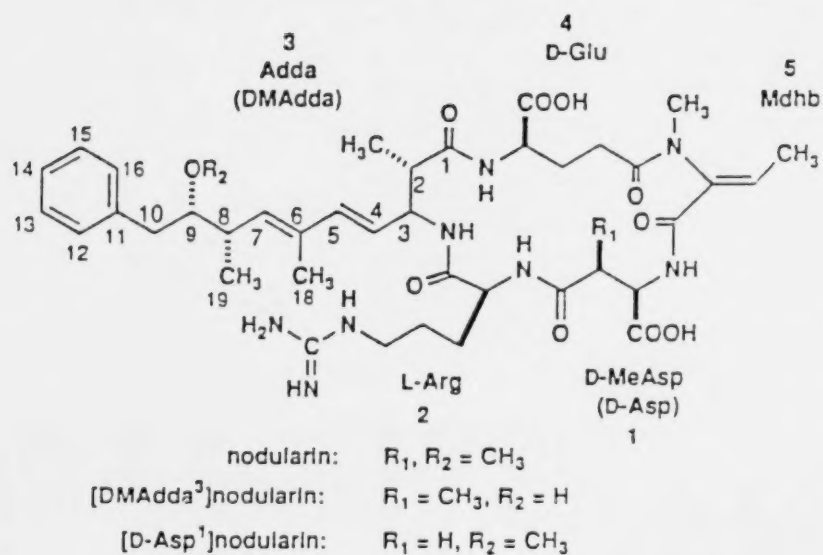


Figure 5. Chemical structure of the hepatotoxic pentapeptide, nodularin with two associated toxic analogues. This toxin is produced by the brackish water cyanobacterium *Nodularia spumigena* (from Rinehart et al. 1994).

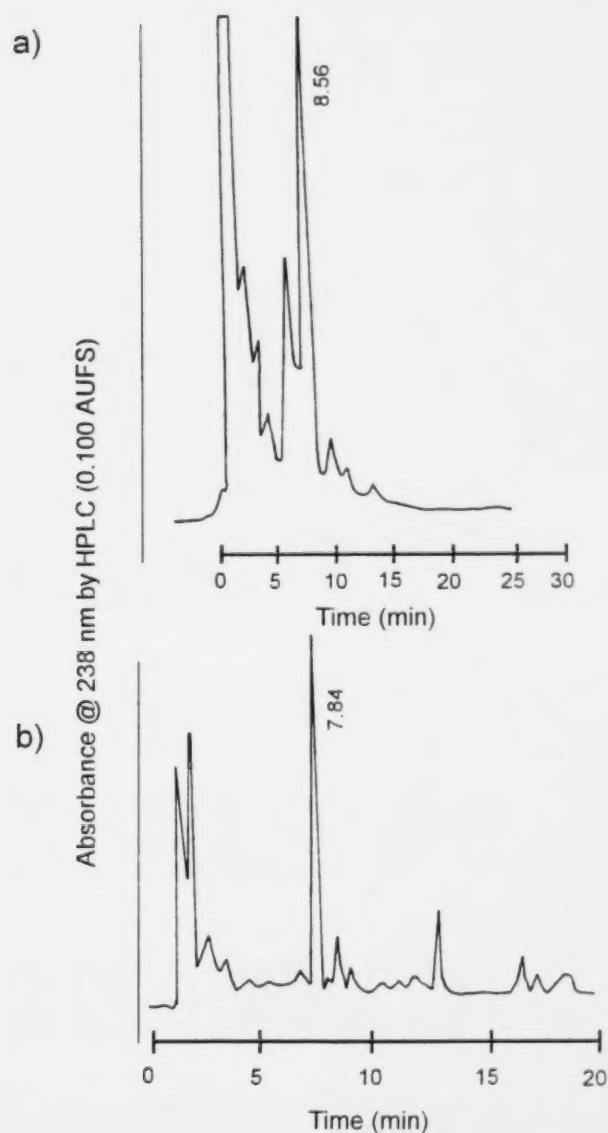


Figure 6. HPLC chromatograms of phytoplankton collected from Driedmeat Lake (11 August 1994) that were analyzed by a) isocratic separation (10mM ammonium acetate: acetonitrile, 80:20%, v/v) and b) gradient separation (20-30% acetonitrile in 10mM ammonium acetate in 10 min, then 20% acetonitrile from 10 to 20 min). Retention time of microcystin-LR in a) is 8.56 min, and in b) is 7.84 min. Both analyses were run on a NovaPak C-18 column at a flow rate of 1.0 mL min^{-1} with UV detection at 238nm.

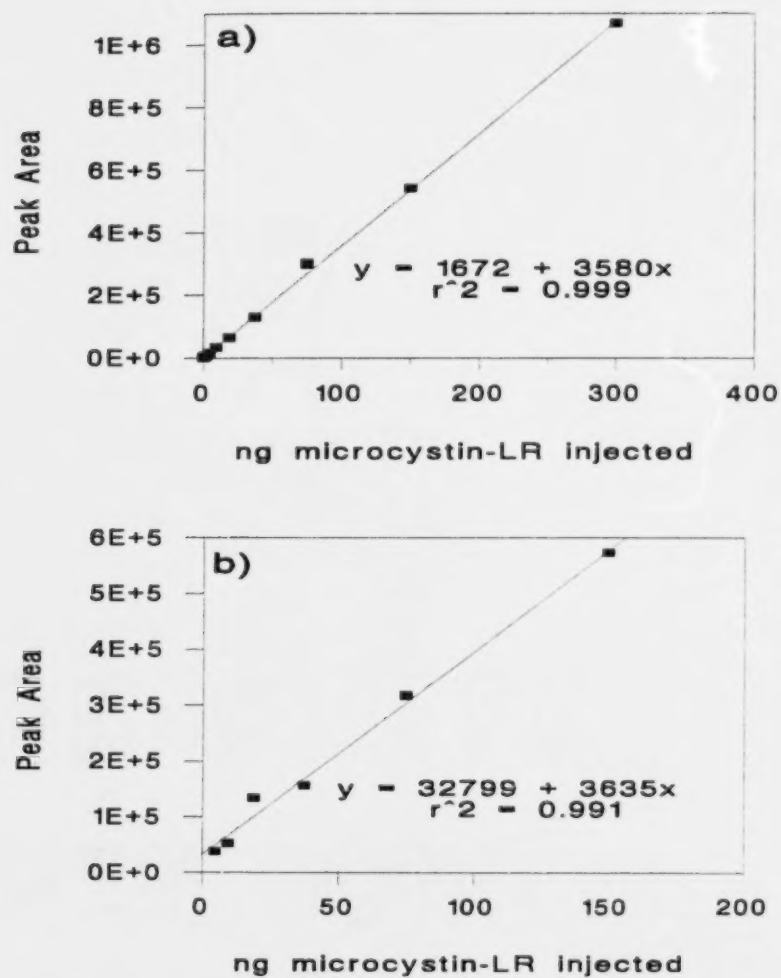


Figure 7. HPLC calibration curves for a) purified microcystin-LR standard, and b) an extract of phytoplankton collected from Coal Lake, 13 June 1994, originally lacking detectable microcystin-LR, but spiked with differing concentrations of purified microcystin-LR. Both curves are based on linear gradient separation (20 to 30% acetonitrile in 10 min, then 20% acetonitrile from 20 to 30 min).

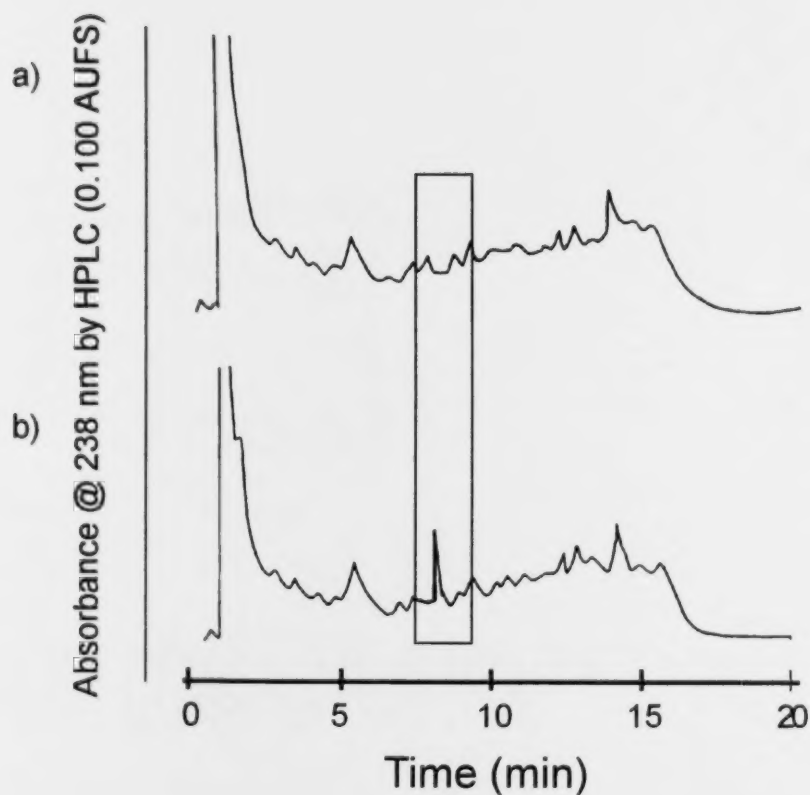


Figure 8. HPLC chromatograms of a) phytoplankton sample from Coal Lake, 13 June 1994, analyzed by linear gradient separation (20 to 30% acetonitrile in 10 min, then 20% acetonitrile from 10 to 30 min) and containing non-detectable microcystin-LR (lacking microcystin-LR peak), and b) above sample spiked with $0.468 \mu\text{g mL}^{-1}$ microcystin-LR standard, equivalent to $9.4 \text{ ng microcystin-LR injection}^{-1}$. Box indicates retention time window of microcystin-LR (8.53 min).

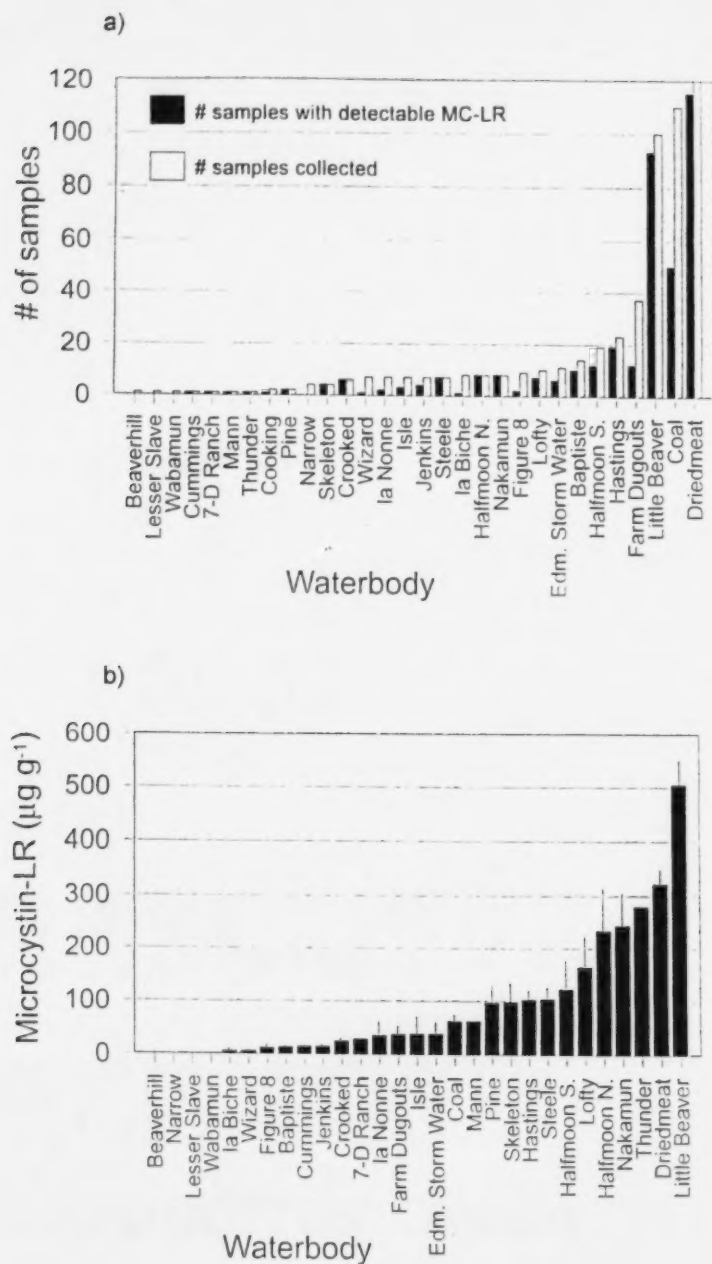


Figure 9. Occurrence of microcystin-LR in Alberta water bodies from 1990 to 1994. A) Number of phytoplankton samples collected and analyzed by HPLC for microcystin-LR and number of samples containing detectable ($> 1 \mu\text{g g}^{-1}$ phytoplankton biomass) microcystin-LR. B) Mean concentration of microcystin-LR in phytoplankton collected from Alberta waterbodies from 1990 to 1994. Vertical bars are standard error of the mean.

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Chapter 3

Occurrence and toxicological evaluation of cyanobacterial toxins in Alberta lakes and farm dugouts¹

Introduction

Many drinking water supplies throughout the world experience periodic massive surface accumulations (blooms) of cyanobacteria. These blooms may contribute significantly to a wide range of water-related problems including summer kills of valuable sportfish, unpalatability of finished drinking water and contribution to trihalomethane formation during water chlorination (Palmstrom et al. 1988). Some species of bloom-forming cyanobacteria produce water soluble neuro- and hepatotoxins which are released into the water when a cyanobacterial bloom dies. Farmers experience economic losses when livestock die after consuming either bloom material or water containing cyanobacterial toxins. These toxins may also pose a serious health hazard to humans (Lawton and Codd 1991).

Incidents of toxic blooms of cyanobacteria occur worldwide (Carmichael et al. 1985) and have been responsible for poisoning and death of domestic cattle, wildlife and pets (Schwimmer and Schwimmer 1968). Furthermore, toxic blooms have been causally related to some human illnesses (Falconer et al. 1983). The extent of toxic bloom occurrence in Canada is unknown, although several toxic blooms have been reported in western Canada (e.g., McLeod and Bondar 1952; Dillenberg and Dehnelt 1960; Gorham 1964; Carmichael and Gorham 1978; 1981).

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Three bloom-forming species are likely responsible for the majority of toxic freshwater blooms in western Canada: *Aphanizomenon flos-aquae* (Li) Ralfs, *Anabaena flos-aquae* (Lyngb.) de Breb. and *Microcystis aeruginosa* Kutz. emend Elenkin. *Aphanizomenon flos-aquae* and *An. flos-aquae* may both produce potent neurotoxins. Anatoxin-a (Fig. 1a), produced by *An. flos-aquae*, is a secondary amine which acts as a neuromuscular blocking agent causing paralysis of skeletal muscle resulting in death due to respiratory arrest in as little as 5 min (Carmichael et al. 1985). *Anabaena flos-aquae* has also been reported to produce a slower-acting (1-2 h) peptide hepatotoxin, identified as microcystin-LR (MC-LR, Fig. 1b), one of several peptide hepatotoxins produced by *M. aeruginosa* (Krishnamurthy et al. 1989). Harada et al. (1991) have also reported the presence of four hepatotoxins from *An. flos-aquae* NRC 525-17. Reports of toxic blooms of *M. aeruginosa* are more widespread, perhaps owing to the cosmopolitan distribution of the species. *Microcystis aeruginosa* produces at least nine different cyclic peptide hepatotoxins (termed microcystins), each composed of 7 amino acids (Botes et al. 1985; Carmichael 1988). The structures of the two hepatotoxins, MC-LR and-RR, monitored in this study are depicted in Figs. 1b and c. Ingestion by animals of toxic bloom material or water containing microcystins may result in irreversible damage to the liver and may cause death from hemorrhagic shock (Falconer et al. 1981).

Materials and Methods

Survey of water bodies - Fourteen water bodies in north and central Alberta (Fig. 2; Table 1) were sampled in 1990; in all, eight lakes and six farm dugouts. Selection of the lakes was based on their proximity to Edmonton, their history of cyanobacterial blooms and/or their use as municipal drinking water supplies for towns and cities, campgrounds and cottage owners. The dugouts were chosen because of their use as rural drinking water supplies. Three of the lakes (Driedmeat, Coal and Little Beaver) were subject to more detailed study (being sampled up to 2 times per week) from 31 July to 24 October 1990, while the remaining lakes were surveyed only once or sampled at irregular

intervals during this period. The dugouts were sampled at either 1- or 2-week intervals from 23 May to 21 August 1990. Bloom material was collected for toxin analyses only on those dates when enough (approx. 1 g dry weight) algae could be collected for toxin analysis by HPLC or GC-MS.

Water samples from all sources were collected on each sampling date and analyzed for various limnological parameters. Integrated surface water samples (i.e., 0 to a depth of 2.5 m) were collected from Driedmeat and Coal lakes with weighted tygon tubing fitted with a one-way valve. Surface (i.e., 0 to 10 cm) grab water samples were collected from Little Beaver Lake. Surface water temperature and Secchi depth were recorded at each lake. Water samples were analyzed for total phosphorus (TP) and total dissolved phosphorus (TDP) based on the persulfate digestion method of Menzel and Corwin (1965) as modified by Prepas and Rigler (1982) although large algae (>250 μm) were not prescreened. Ammonium (NH_4^+) was determined by colorimetric methods on a Technicon AutoAnalyzer II (Stainton et al. 1977). Phytoplankton biomass was estimated as chlorophyll *a* (Chla). Water samples were filtered onto Whatman GF/C filters and 1 mL of magnesium carbonate added to the filter. Chla was extracted from the filter with 95% ethanol and quantified spectrophotometrically as outlined in Winternans and DeMots (1965) and Bergmann and Peters (1980).

In addition to water samples, cyanobacteria were collected from the surface of the surveyed waterbodies and concentrated with a 64- μm plankton net. A small subsample (5 mL) was used for species identification of the dominant cyanobacteria as described by Prescott (1978), while the remainder of the cyanobacteria samples were freeze-dried for subsequent analysis. The relative proportion of each cyanobacteria species in the net plankton was determined and each species reported as a percentage of all cyanobacteria species present in the sample, based on cell counts. In addition, the dominant cyanobacteria in some bloom samples were identified to species but the percent of each species was not evaluated.

Cyanobacterial toxin analysis - Microcystins-LR and -RR analyses involved HPLC with UV detection while GC-MS was used for anatoxin-a analysis. FAB-MS was

used to confirm the molecular mass of the toxic fraction from Coal Lake bloom samples. Mouse bioassays were performed on a subset of the bloom samples to confirm the acute toxicity of the cyanobacteria.

The method of Himberg et al. (1989) was modified slightly, as described by Kenefick et al. (1992) for the GC-MS analysis of anatoxin-a. The method of Harada et al. (1988) was also modified slightly, as described by Kenefick et al. (1992) for the HPLC analysis of the microcystins.

Bloom samples collected from Coal Lake on 10, 16, 26 and 30 August 1990 were freeze-dried, then combined to yield a total biomass of 6.5 g for the FAB-MS confirmation. The toxin extraction procedure of Harada et al. (1988) was used. The extract was subjected to silica gel column chromatography (Kieselgel 60, 230-400 mesh, chloroform:methanol:water (65:35:10), lower layer). Toxin-containing fractions were purified with preparative TLC (Kieselgel 60F₂₅₄, chloroform:methanol (1:1)) yielding about 4 mg of the compound. FAB-MS spectra were performed in a modified AEI/Kratos MS9 double focussing mass spectrometer, equipped with a saddle field atom gun operated with xenon at 7 KV and 1 mA. In each case, a small amount of the sample was taken from the sample vial with the tip of a fine spatula and dissolved in 2 µL of Cleland Matrix (dithiothreitol/dithioerithritol), commonly called "Magic Bullet", previously placed on the probe tip by means of a micropipette. The probe was introduced into the ion source and the sample solution exposed to the action of the atom beam. Five scans were recorded, the first two generally yielding the best spectra, with clear pseudomolecular ions and low levels of chemical noise.

Mouse bioassays - Mouse bioassays were performed on a subset of the bloom samples to 1) confirm the presence of microcystin-LR (MC-LR) identified in bloom samples by HPLC, 2) examine the effect of hepatotoxin dosage on liver damage in mice, and 3) screen for hepatotoxins other than MC-LR and -RR present in the bloom samples. The extraction of toxins from the bloom material and subsequent injection into mice followed two methods. One method involved treatment of approximately 25 mg of

freeze-dried algal biomass with 10 mL 0.9% NaCl solution followed by sonication for 1 min. To confirm the toxicity of selected bloom samples to mice and also to examine the effect of toxin dosage on liver damage in mice, bloom extracts, including the ruptured cells, were injected intraperitoneally (i.p.) into five to 12 replicate male CD1 mice (ca. 20 to 25 g) at an extract dose of 10 μ L per gram of body weight. These samples were termed "whole cell extracts". A second set of mouse bioassays were performed to screen a larger proportion of the bloom samples for toxicity. Only two to three replicate mice were used in these mouse bioassays. Initial 25mg aliquots of bloom biomass were suspended in 1 mL 0.9% NaCl, sonicated and then centrifuged for 40 min at 4000g. The supernatants were centrifuged a second time for 10 min at 12000g. The final supernatants were termed "centrifuged samples" and were injected into male ICR Swiss mice. Consequently, mice in the centrifuged sample bioassays received ten times the mass of algae as mice in the whole cell extract bioassays. In all mouse bioassays, control mice received saline injections only.

All mice were observed continuously for 6 h for signs of hepatotoxicity. Mice surviving 6 h were periodically monitored for the next 18 h. Mice dying post-injection were necropsied and gross pathologic changes in the livers were noted. Livers were removed from mice, weighed and examined for gross pathology. Liver weights were expressed as a percentage of the body mass of the mouse. Mice surviving after injection were sacrificed and the livers removed and treated as above. To confirm that the probable cause of toxicity was due to MC-LR, a series of mice were also injected with purified MC-LR standard at doses of 25, 50 and 100 μ g kg^{-1} .

Results

Characteristics of Driedmeat, Coal and Little Beaver lakes - The three lakes studied in more detail serve as drinking water supplies for communities in Alberta. Driedmeat Lake, the drinking water supply for the city of Camrose, and Coal Lake, the drinking water supply for the city of Wetaskiwin, are shallow and of moderate size (Table 2) both with a similar high nutrient regime (TP, TDP, NH_4^+) and are classified as

hypereutrophic based on phosphorus concentration and historic chlorophyll *a* data (Mitchell and Prepas 1990). Little Beaver Lake, the drinking water supply for the village of Ferintosh, is much smaller and more shallow than the other two lakes and had the highest nutrient and chlorophyll *a* concentrations during much of the three month study (Table 2). TP and Chl *a* concentrations throughout the 1990 sampling period in Driedmeat Lake in particular, were lower than historical concentrations (Tables 1 and 2). No historical data are available for Little Beaver Lake.

All three lakes had hardwater (200 to 350 mg L⁻¹ CaCO₃) and ranged in salinity from fresh (conductivity 500 µS cm⁻¹, Driedmeat and Coal lakes) to moderately saline (conductivity 1500 µS cm⁻¹, Little Beaver Lake). The ionic composition of the surface water in all three lakes was dominated by sodium and bicarbonate. Surface water temperature declined from maximum values of 22 to 25 °C in August to 5 to 7 °C in October in all three lakes. Little Beaver Lake had the warmest water in August and cooled more rapidly than the other two lakes in October.

Average August to October 1990 surface Chl *a* concentration was 1.5- to 3-fold higher in Little Beaver Lake than in Driedmeat or Coal lakes (Table 2) and was more variable in Little Beaver Lake (Figs. 3a, b, and c). Chl *a* patterns were distinct in the three lakes over the three months of this study. Chl *a* concentration was highest in Little Beaver Lake (up to 216 µg L⁻¹) during August and the beginning of September and dropped to 20 to 35 µg L⁻¹ for the rest of September and all of October. Chl *a* concentration in Coal Lake peaked in mid September (approx. 40 µg L⁻¹) while it generally increased throughout the sampling period in Driedmeat Lake, reaching 25 µg L⁻¹ by the last sampling date.

The surface blooms of all three lakes were dominated by cyanobacteria, most notably *Microcystis aeruginosa*, *Aphanizomenon flos-aquae* and *Gomphosphaeria* spp. (Table 3). *Anabaena flos-aquae* was rare in the surface blooms of these lakes. In August and September *M. aeruginosa* was more dominant in the algal biomass in both Coal and Driedmeat lakes (up to 80 to 90%) than in Little Beaver Lake (usually <10%; Table 3). The other lakes which were surveyed only once during 1990 (i.e. Hastings, Cooking, Nakamun, Lac la Nonne and Isle) had TP concentrations from 83 to 288 µg L⁻¹ and Chl *a*

concentrations ranging from 27 to 40 $\mu\text{g L}^{-1}$ in their surface (i.e. 0 to 2.5 m) water (data not shown).

Occurrence of Microcystins-LR and -RR and anatoxin-a - Detectable concentrations of microcystin-LR (MC-LR) were found in 37 out of 39 bloom samples collected (i.e., 95%) from the eight lakes surveyed (Table 3). *Microcystis aeruginosa* was present in 34 of these 37 microcystin-laden samples, leaving only three bloom samples containing detectable concentrations of MC-LR without the presence of *M. aeruginosa* (Table 3). Bloom samples from the two lakes (Cooking Lake and Lac la Nonne) which had no detectable concentration of MC-LR, also had either an absence or a low proportion of *M. aeruginosa*. Therefore, the presence of *M. aeruginosa* in a bloom was a relatively consistent indicator that MC-LR would be detectable.

Of the more intensively studied lakes, Coal Lake had the highest proportion of *M. aeruginosa* in its algal blooms and the proportion of *M. aeruginosa* in the bloom material was related to the concentration of MC-LR (Fig. 4; $r=0.90$, $P < 0.001$, $df=11$). In the other two lakes studied more intensively, there was no apparent relationship (Driedmeat Lake, $r=0.15$, $P > 0.1$, $df=3$, Little Beaver Lake, $r=0.03$, $P > 0.1$, $df=10$). *Microcystis aeruginosa* was rare in samples collected from farm dugouts and MC-LR was not detected in any of the material collected from these dugouts. Several colonies of *M. aeruginosa* were isolated from the Pretzlaff dugout and cultured in the laboratory. These isolates subsequently produced high concentrations (204 to 446 $\mu\text{g g}^{-1}$) of MC-LR.

Neither MC-RR nor anatoxin-a were detected in fresh bloom material from any of the lakes or dugouts. However, anatoxin-a was detected (63 $\mu\text{g g}^{-1}$) in a laboratory culture of an isolate of *An. flos-aquae* from the Frey dugout.

Confirmation of MC-LR by FAB-MS - To confirm the presence of MC-LR in Coal Lake algae, both an isolate from a composite toxin sample and a standard sample were subjected to mass spectrometric analysis. The pseudomolecular ion region of the FAB mass spectra of the toxin isolated from the combined samples of bloom material from Coal

Lake and that of the standard sample of MC-LR are shown in Fig 5a+b, respectively. In both, clear pseudomolecular ions $(M+H)^+$ at m/z 995 are observed and the intensities and isotopic patterns are practically identical. No significant fragmentation occurred, which is characteristic of cyclic peptides. In contrast, open chain peptides would show some fragmentation indicating the sequence of amino acids present in the molecule. The only other significant peaks in the spectra were from the Magic Bullet matrix. The presence of a relatively high concentration of sodium salts is very commonly observed in FAB-MS, especially in natural product samples. In the case of the isolated sample, additional pseudomolecular ions formed by association with sodium $(M+Na)^+$ at m/z 1017, $(M+Na)^+Na$ at m/z 1040 and $(M+Na)^+Na_2$ at m/z 1063 were observed. These additional ions reinforce the confirmation of the molecular mass of 994 g mol^{-1} , characteristic of MC-LR.

Mouse Bioassays - A total of 24 freeze-dried cyanobacterial bloom samples from six of the eight lakes and five of the six dugouts were used in the mouse bioassay experiments. Bloom extracts were injected i.p. into mice giving a dose range of 0 to $600 \mu\text{g MC-LR kg}^{-1}$ corresponding to doses up to 12 times the reported LD_{50} value ($LD_{50} = 50 \mu\text{g kg}^{-1}$ in mice; Carmichael 1988). A large proportion of the bloom samples either induced signs of poisoning in the mice or caused death at the doses injected (Table 3).

Signs of poisoning in mice were similar whether injected with 1) sublethal or lethal doses of the lake bloom extracts containing MC-LR, 2) whole cell or centrifuged extracts, or 3) the toxin standard. Signs appeared after a 20 to 30 minute latent period, post-injection and included piloerection, unprovoked episodes of leaping, dragging of the hind limbs while walking, progressing to lethargy, pallor of the eyes, ears and tail and gradual ataxia. Signs just prior to death were complete loss of mobility and shallow, very slow breathing. Mice injected with extracts of three of the six dugout samples (Helbig Upper 31-May, F. Lambert 5-July, and Pretzlaff 7-Aug.) also displayed signs of poisoning including lethargy and pallor of the extremities. Blooms from these samples were dominated by either *M. aeruginosa* (Pretzlaff) or *An. flos-aquae* (Helbig Upper and F.

Lambert). Mice injected with saline (controls) remained active and showed none of the above signs.

The mortality of mice clearly depended on the dose of algal extract administered. Mice given lethal doses (50 to 600 $\mu\text{g kg}^{-1}$) generally died in 1 to 2 h post-injection and there was no relationship between the dose of MC-LR injected and the time to death. Generally, mice injected with lake bloom extracts corresponding to a dose of 50 $\mu\text{g MC-LR kg}^{-1}$ or more died. Only two mice died when injected with an extract at a dose corresponding to less than 50 $\mu\text{g kg}^{-1}$ (Table 4). These findings are consistent with the results of the mice which were injected with the purified toxin; none of the 4 mice died with a dose of 25 $\mu\text{g kg}^{-1}$, 9 out of 10 died with 50 $\mu\text{g kg}^{-1}$ and 5 out of 5 with 100 $\mu\text{g kg}^{-1}$ (Table 4). Levels of mortality differed among groups of mice given an LD_{50} (i.e., 50 $\mu\text{g kg}^{-1}$) dose of purified toxin, whole cell extracts or centrifuged extracts. Less than 50 % mortality occurred in mice when injected with a LD_{50} dose of the centrifuged extracts while nearly 100 % mortality occurred in mice injected with a LD_{50} dose of either the whole cell extracts or the purified toxin. No mice died after injection with extracts from the dugout blooms.

Injection of the mice with either the purified toxin or lake bloom extracts containing MC-LR resulted in similar gross pathological changes to the liver. Lethal doses resulted in livers that were moderately enlarged and dark red. Mottling of the liver, consistent with zonal necrosis was also evident. The severity of enlargement, discoloration and mottling was much less in mice given a sublethal dose. In addition, mice injected with extracts from three of the six dugouts (Helbig Upper 31 May, F. Lambert 5 July, and Pretzlaff 7 Aug.) had livers that were slightly enlarged, mottled and dark red. MC-LR was not detected in these blooms by HPLC. The livers of mice injected with bloom extracts from other dugouts appeared similar to saline controls. In addition to liver damage, multifocal to diffuse periarterial and arterial hemorrhage was seen in the lungs of some of the mice injected with the Coal Lake bloom extracts and the purified MC-LR. The severity of hemorrhage increased with increasing doses of MC-LR. Mice injected with saline did not have these hemorrhages. All other organs in the MC-LR-dosed mice appeared normal, except for a notable lack of blood in the organs.

Liver weight of the mice increased with increased dose of MC-LR (Table 4). Mice injected with extracts from Helbig Upper, F. Lambert and Pretzlaff bloom samples had mean liver weights of 8.3, 7.2 and 8.3% respectively, significantly higher ($P < 0.01$, t -test) than the mean ratio of 6.6% for control mice (Table 5). Injection with extracts from the other dugouts gave no significant increase in liver weight.

Discussion

Reports of toxic blooms of freshwater cyanobacteria worldwide are appearing more frequently in the water quality literature. Progressive eutrophication of lakes through land use changes may result in elevated nutrient loadings to the lakes and may be causing an increase in bloom frequency (Codd and Bell 1985). My survey found a high incidence of cyanobacteria in lakes and farm dugouts in Alberta, waters which are regarded as being eutrophic to hypereutrophic (Mitchell and Prepas 1990). The presence of *M. aeruginosa* in a bloom from the lakes we surveyed was usually indicative that microcystin-LR (MC-LR) would be detectable by HPLC. The cosmopolitan distribution of *M. aeruginosa* suggests a cosmopolitan distribution of its associated toxins. A recent survey of 102 water bodies by Repavich et al (1990) revealed that approximately 25% of the blooms investigated in Wisconsin waters were toxic based on mouse bioassays. The toxins in the Wisconsin study were not identified but likely included hepatotoxins and neurotoxins.

The results obtained from the three lakes chosen for detailed study indicated substantial temporal variation in the concentration of MC-LR present in the bloom material. The factors (both environmental and intrinsic) which control toxin production in cyanobacteria are poorly understood. The factors identified as being important in the formation of cyanobacterial blooms (e.g., nutrient concentrations, water temperature, light, wind conditions, etc.) also appear to be important in toxin production, although the exact mechanisms are not known (Carmichael 1986). For example, Wicks and Thiel (1990) monitored hepatotoxin concentration in the algal biomass in Hartbeespoort Dam, South Africa over a 2.5-yr period. Hepatotoxin concentration in the algae was strongly,

positively correlated to primary productivity per unit chlorophyll *a*, solar radiation, surface water temperature, pH and % oxygen saturation while weakly, negatively correlated to chlorophyll *a* and soluble reactive phosphorus concentration in the water. The concentration of MC-LR in the algal biomass in Coal Lake during 1990 was also strongly correlated with water temperature, phosphorus concentration and Secchi depth but not with chlorophyll *a* (Table 6). The lack of correlation between the concentration of MC-LR in the bloom and chlorophyll *a* concentration in Coal Lake may be due to the contribution to the total chlorophyll *a* concentrations of other algal species, which do not produce MC-LR. If chlorophyll *a* concentrations are weighted for the proportion of *M. aeruginosa* in the bloom, then chlorophyll *a* concentration is strongly correlated to MC-LR concentration in the bloom ($r=0.70$, $P=0.01$, $df=10$).

Toxin production is likely a function of many interacting variables and correlations drawn between such environmental variables and toxin production (as in this study and others) which are based on limited sample size, should be interpreted with caution. For example, Carmichael and Gorham (1981) found substantial spatial variation in species composition of a bloom and toxin concentration over short (i.e., 10 m) distances in Hastings Lake, Alberta. They speculated that part of the variation in toxin content of the bloom could be attributed to differing proportions of toxic and non-toxic strains and/or species within the bloom. Variability in the proportions of genetically distinct strains or populations of a particular algal species may also give spatial variation in the toxicity of a bloom. This may also explain the differences through time with respect to toxin concentration in each lake and those differences seen between Driedmeat, Coal and Little Beaver Lakes.

The FAB-MS spectra of the toxin isolated from the pooled samples from Coal Lake confirmed that the algal extracts contained MC-LR. The spectra obtained compared very favorably with that obtained with the purified MC-LR standard and documented spectra of MC-LR (Krishnamurthy et al. 1989; Harada et al. 1988).

Bioassays confirmed the toxicity of MC-LR to mice. All mice injected with extracts of bloom material containing MC-LR showed the signs of toxicity that are characteristic of microcystins (Elleman et al. 1978; Carmichael et al. 1985). In particular,

I observed enlargement (engorgement) and mottling of the liver. This pathological characteristic is caused by the destruction of the hepatic sinusoidal lining, resulting in massive hemorrhage and pooling of blood (Hooser et al. 1989). Adams et al. (1988) using ^{51}Cr -labelled erythrocytes, found that as much as 44% of the total blood volume of the body could end up pooling in the liver, as compared to 4% in control mice injected with saline only. This increased blood volume resulted in the weight increase observed in the livers of affected mice in my study. An increased liver weight is a characteristic feature of intoxication by *M. aeruginosa* (Falconer et al. 1981; Brooks and Codd 1987). Mice injected with extracts of blooms not containing MC-LR, *M. aeruginosa* or *An. flos-aquae* displayed none of these signs, with two notable exceptions.

Mice injected with the bloom extract from Little Beaver Lake (4 October) had a mean liver weight ratio of 7.7%, slightly higher than the control mice livers. It is likely that a small proportion of *M. aeruginosa* was present in the sample but was not observed during species identification, given the detection of MC-LR by HPLC. Mice injected with a bloom extract from Cooking Lake (31 July, Table 3) containing neither *M. aeruginosa* nor a detectable concentration of MC-LR, displayed clinical signs characteristic of hepatotoxicity. This bloom was dominated by *Microcystis flos-aquae*. This bloom may have produced a hepatotoxin for which I had no standard. Several peaks were present in the HPLC chromatogram for this sample. Carmichael et al. (1988) have documented a toxic bloom (based on mouse bioassay) of *Microcystis flos-aquae* in central China. However, the structure of the hepatotoxin has yet to be reported.

No relationship was found between the dose of MC-LR injected and the time to death in this study. All mice given lethal doses died in 1 to 2 h. The apparent lack of time dependence is likely due to the rapid nature of the action of MC-LR. Our results are consistent with the findings of Siegelman et al. (1984) whereby supra-lethal doses (four times the LD_{50}) did not affect survival time.

Death in mice depended on the dosage of MC-LR injected. The LD_{50} of MC-LR in mice by i.p. injection is reported to be $50 \mu\text{g kg}^{-1}$ (Carmichael 1988). In my study, a dose of $50 \mu\text{g kg}^{-1}$ consistently resulted in near-100% mortality indicating an LD_{50} of the material used in this study (both cell extracts and the purified toxin) somewhere between

25 and 50 $\mu\text{g kg}^{-1}$. The LD_{50} of MC-LR has been reported to be variable in mice, perhaps depending on the strain and age of mice used. LD_{50} values of MC-LR from other studies of mice range from 30 (Stoner et al. 1989) to 125 $\mu\text{g kg}^{-1}$ (Brooks and Codd 1987). For comparison, an LD_{50} of less than 5 mg kg^{-1} has been classified as "supertoxic" (Klaassen et al. 1986). The LD_{50} of 2,3,7,8- tetrachlorodibenzodioxin (2,3,7,8-TCDD), an infamous hepatotoxin and the most acutely toxic of the polychlorinated dioxins and furans ranges from 0.6 $\mu\text{g kg}^{-1}$ for guinea pigs to 5015 $\mu\text{g kg}^{-1}$ for hamsters (Government of Canada 1990). The LD_{50} of 2,3,7,8-TCDD in laboratory mice is 100 to 600 $\mu\text{g kg}^{-1}$ (EPA 1985), a less toxic range than that reported for MC-LR with mice.

The occurrence of cyanobacterial toxins in drinking water supplies may represent a serious health hazard to humans. Lifetime consumption of low concentrations of MC-LR may contribute to chronic liver problems such as necrosis or present a risk of liver tumors (Falconer et al. 1988; Falconer 1991). Conventional water treatment processes including alum flocculation, sand filtration and chlorination are ineffective at removing MC-LR from water (Hoffman 1976; Himberg et al. 1989). Ozonation and activated carbon (powdered or granular) are effective at MC-LR removal (Falconer et al. 1989; Himberg et al. 1989) providing the systems are operating under optimal conditions. However, small rural communities may use relatively simple water treatment technology and it is unlikely that they would be able to operate more advanced technology in an optimum manner.

Table 1. Historical euphotic zone concentrations of total phosphorus (TP) and chlorophyll *a* (Chla) in the lakes and farms dugouts sampled in the 1990 survey. Concentrations are mean values for samples taken from May to September in the years indicated. Standard error is in parentheses.

Water body	Year	TP ($\mu\text{g L}^{-1}$)	Chla ($\mu\text{g L}^{-1}$)
<i>Lakes</i> ^a			
Hastings	1981	156 (6)	75 (33)
	1987	116 (2)	73 (52)
Cooking	1983	232 (19)	83 (15)
	1987	270 (11)	84 (11)
Coal	1984	176 (50)	44 (14)
	1985	c	43 (12)
Driedmeat	1984	453 (134)	87 (29)
Little Beaver	c	c	c
Nakamun	1982	97 (4)	55 (5)
	1983	80 (11)	34 (18)
la Nonne	1988	168 (29)	56 (18)
Isle	1983	94 (24)	39 (11)
	1984	109 (33)	39 (6)

Table 1 (continued).

Water body	Year	TP ($\mu\text{g L}^{-1}$)	Chla ($\mu\text{g L}^{-1}$)
<i>Dugouts</i> ^b			
Frey	1989	61 (11)	10 (3)
	1990	106 (16)	27 (8)
Screpnek	1989	36 (4)	3 (0.8)
	1990	52 (3)	10 (2)
F. Lambert	1898	107 (22)	26 (10)
	1990	170 (29)	35 (9)
Helbig Beaver	1990	114 (3)	26 (8)
Helbig Upper	1990	55 (5)	8 (2)
Prezlaff	1987	348 (3)	36 (23)
	1988	166 (18)	14 (0.6)
	1989	231 (14)	22 (4)
	1990	546 (52)	28 (9)

^a Data obtained from Mitchell and Prepas (1990).

^b 1990 data from this survey, all other dugout data from Prepas (unpublished)

^c no data

Table 2. Limnological characteristics of the three lakes subjected to more detailed study. Water chemistry (total phosphorus [TP], total dissolved phosphorus [TDP], ammonium [NH_4^+]) and chlorophyll *a* (Chla) are mean surface concentrations (0 to 2.5 m in Driedmeat and Coal lakes, 0 to 10 cm in Little Beaver Lake) for the months indicated in 1990. Standard error is in parentheses. pH ranged from 8.1 to 8.9 in the three lakes.

Parameter	Units	Month	Lake		
			Driedmeat	Coal	Little Beaver
Surface area ^a	km ²		17	11	0.75 ^b
Mean depth ^a	m		2.2	3.5	^c
Max. depth ^a	m		3.7	5.5	2.0 ^b
Volume ^a	$\times 10^6 \text{ m}^3$		42	39	^c
TP	$\mu\text{g L}^{-1}$	August	244 (41)	178 (24)	436 (25)
		September	150 (57)	157 (9)	416 (27)
		October	111 (6)	104 (10)	317 (44)
TDP	$\mu\text{g L}^{-1}$	August	186 (35)	115 (21)	315 (33)
		September	115 (12)	78 (8)	363 (30)
		October	315 (13)	45 (7)	236 (26)
NH_4^+	$\mu\text{g L}^{-1}$	August	201 (99)	133 (72)	1145 (512)
		September	75 (46)	134 (45)	1889 (546)
		October	39 (15)	116 (30)	2220 (99)
Chla	$\mu\text{g L}^{-1}$	August	11 (4)	32 (6)	94 (32)
		September	11 (1)	34 (2)	14 (3)
		October	22 (3)	16 (3)	21 (5)
Secchi depth	m	August	1.6 (0.4)	1.6 (0.2)	0.7 (0.1)
		September	2.3 (0.2)	1.3 (0.0)	>1.5
		October	1.2 (0.0)	1.4 (0.2)	1.6 (0.1)

^a Morphometric data obtained from Mitchell and Prepas (1990)

^b F. Jackson. Village of Ferintosh, pers. comm. (1990)

^c Unknown

Table 3. Water bodies sampled during 1990 with their associated cyanobacterial bloom composition and toxicity to mice. Mouse bioassay results shown are for toxicity screening with "centrifuged samples" only and male ICR Swiss mice. Mouse bioassays for farm dugout samples are expanded in Table 5.

Water body	Sampling date	Dominant cyanobacteria ^a M.:Aph.:An.:G.:O	Microcystin-LR in bloom (µg g ⁻¹) ^b	Mouse bioassays	
				Microcystin-LR dose (µg kg ⁻¹) ^c	Pathological effects ^d
<i>Lakes</i>					
Hastings	31 July	Y:Y:Y:Y:N	8		
Cooking	31 July	N:N:N:N:Y	ND	0	+
Coal	1 August	48:50:0:2:0	189		
	10 August	85:13:0:2:0	507	526	++
	16 August	90:10:0:0:0	490		
	22 August	80:5:0:15:0	387		
	26 August		605	350	++
	30 August	40:30:0:30:0	418	175	++
	10 September	10:39:0:51:0	170		
	13 September	18:28:0:54:0	124	60	++
	17 September	18:30:0:52:0	219	100	++
	20 September	6:54:0:40:0	47		
	24 September	3:62:0:35:0	98		
	27 September	Y:Y:N:Y:N	65		
	1 October	2:40:0:58:0	179		
	4 October	2:13:0:85:0	53	25	+
	11 October	0:17:0:83:0	6		

Table 3. (continued).

Water body	Sampling date	Dominant cyanobacteria ^a M.:Aph.:An.:G.:O	Microcystin-LR in bloom ($\mu\text{g g}^{-1}$) ^b	Mouse bioassays	
				Microcystin-LR dose ($\mu\text{g kg}^{-1}$) ^c	Pathological effects ^d
Driedmeat	1 August	45:50:5:0:0	87	50	++
	10 August	Y:Y:Y:Y:N	17		
	16 August	80:20:0:0:0	177	100	++
	10 September	40:21:26:13:0	372	365	++
	13 September	33:55:9:3:0	245	160	++
	17 September	1:90:7:2:0	199		
Little Beaver	1 August	Y:Y:N:N:N	8		
	10 August	5:95:0:0:0	24		
	16 August	5:95:0:0:0	12	8	+
	22 August	5:95:0:0:0	34	20	+
	30 August	10:90:0:0:0	53		
	10 September	17:83:0:0:0	20		
	13 September	6:94:0:0:0	21		
	20 September	5:95:0:0:0	209	135	++
	24 September	5:95:0:0:0	435	600	++
	27 September	5:95:0:0:0	74	50	++
	1 October	1:92:0:0:7	30		
	4 October	0:100:0:0:0	25	16	+
	11 October	0:100:0:0:0	4		
Nakamun	2 August	Y:N:N:Y:Y	39	25	+
la Nonne	2 August	Y:Y:N:Y:N	ND	0	+
Isle	19 August	95:5:0:0:0	244		

Table 3. (continued).

Water body	Sampling date	Dominant cyanobacteria ^a M.:Aph.:An.:G.:O	Microcystin-LR in bloom (µg g ⁻¹) ^b	Mouse bioassays	
				Microcystin-LR dose (µg kg ⁻¹) ^c	Pathological effects ^d
<i>Dugouts</i>					
Frey	23 May	N:N:Y:N:N	ND		
	6 June	N:Y:Y:N:Y	ND		
	20 June	N:Y:Y:N:N	ND		
Screpnek	5 June	N:Y:Y:N:Y	ND		
	16 July	N:Y:N:N:Y	ND		
F. Lambert	6 June	N:Y:Y:N:N	ND		
	20 June	N:Y:Y:N:N	ND		
	5 July	N:Y:Y:N:N	ND		
Helbig Beaver	21 August	N:Y:N:N:Y	NA		
Helbig Upper	31 May	N:N:Y:N:Y	ND		
Pretzlaff	30 July	N:Y:N:N:N	NA		
	7 August	Y:Y:N:N:N	ND		
	14 August	Y:Y:N:N:N	ND		

^a Refers to the % of each cyanobacterial species in the bloom material based on the following format: M = *M. aeruginosa*:Aph = *Aph. flos-aquae*:An = *An. flos-aquae*:G = *Gomphosphaeria* sp.:O = other. Note: species from some of the bloom samples were identified but the % of each species was not estimated. In these samples, the species present are indicated by a "Y", species absent are indicated by a "N".

^b Identification of microcystin-LR in the bloom biomass based on HPLC retention time only.

^c Dose as μg microcystin-LR based on HPLC measured concentration in the algal biomass per kg of mouse body weight.

^d Results from mouse bioassays are presented in the following format: ++, mice with signs of intoxication, increased liver weight and died, + mice with signs of intoxication/or increased liver weight only.

NA = HPLC analysis for toxins was not carried out, ND = not detected by HPLC.

Table 4. Acute exposure mouse bioassays (CD1 mice) with purified microcystin-LR and bloom biomass from Coal Lake. Data presented for Coal Lake and Lac la Nonne are from "whole cell extract" samples.

Toxin source	Microcystin-LR dose ($\mu\text{g kg}^{-1}$) ^d	Number of mice exposed	Mortality (%)	Liver damage (%)	Liver weight (% of body $\pm$ SE)
Purified standard ^a	100	5	100	100	10.2 $\pm$ 0.2
	50	10	90	100	9.6 $\pm$ 0.5
	25	4	0	0	6.7 $\pm$ 0.1
Saline control	0	2	0	0	5.3 $\pm$ 0.6
Coal Lake ^b					
10 August	100	1	100	100	9.3 $\pm$ 0.0
10 August	50	8	87	87	8.8 $\pm$ 0.5
16 August	50	8	87	87	8.7 $\pm$ 0.4
22 August	50	8	100	100	9.3 $\pm$ 0.2
16 August	25	2	100	100	8.5 $\pm$ 0.4
Saline control	0	2	0	0	5.6 $\pm$ 0.3
Lac la Nonne ^c	0	4	0	0	6.0 $\pm$ 0.4

^a Microcystin-LR standard obtained from Dr. W.W. Carmichael.

^b Bloom biomass collected from Coal Lake on the dates indicated.

^c Bloom biomass collected from Lac la Nonne on 2 August and found to contain non-detectable microcystin-LR by HPLC.

^d Dose as μg microcystin-LR based on HPLC measured concentration in the algal biomass per kg of mouse body weight.

Table 5. Acute exposure mouse bioassays with bloom samples from farm dugouts. Data presented are "centrifuged extract" samples and male ICR mice.

Dugout / date	Dose ($\mu\text{g kg}^{-1}$)	Number of mice exposed	Mortality (%)	Liver damage (%)	Liver weight (% of body weight $\pm$ SE)
Screpnek / 16 July	ND ^a	3	0	0	6.8 $\pm$ 0.3
F. Lambert / 5 July	ND	3	0	66	7.2 $\pm$ 0.1
Helbig Beaver / 21 August	ND	2	0	0	6.9 $\pm$ 0.2
Helbig Upper / 31 May	ND	2	0	100	8.3 $\pm$ 0.1
Pretzlaff / 7 August	ND	2	0	100	8.3 $\pm$ 0.2
Saline control	ND	4	0	0	6.6 $\pm$ 0.1

^a ND indicates that dose as $\mu\text{g microcystin-LR per kg of mouse body weight}$ was not measurable based on HPLC detection limit. This corresponded to less than approx. 1 $\mu\text{g per g of dry bloom biomass}$.

Table 6. Summary of correlation analyses for various limnological variables versus concentration of microcystin-LR in the bloom biomass at Coal Lake, 1990.

Variable	Units	<i>r</i>	<i>P</i>	df
Surface water temperature	°C	0.76	<0.01	12
Total Phosphorus	µg L ⁻¹	0.57	0.06	8
Total Dissolved Phosphorus	µg L ⁻¹	0.76	0.01	8
Chlorophyll <i>a</i>	µg L ⁻¹	0.09	>0.50	12
Secchi depth	m	0.76	<0.01	11

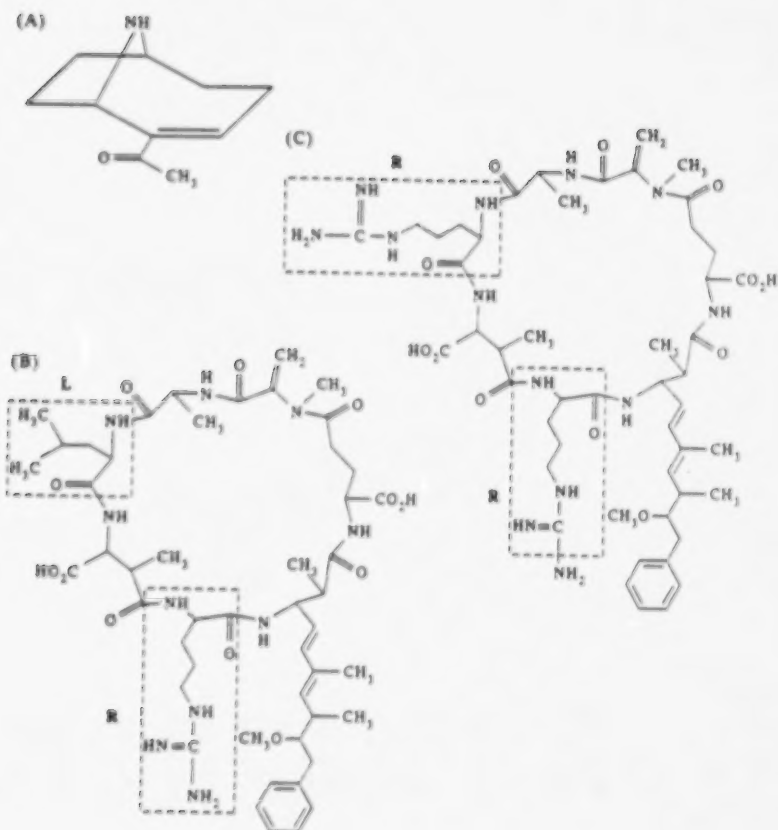


Figure 1. Chemical structures of A) anatoxin-a, B) microcystin-LR and C) microcystin-RR.

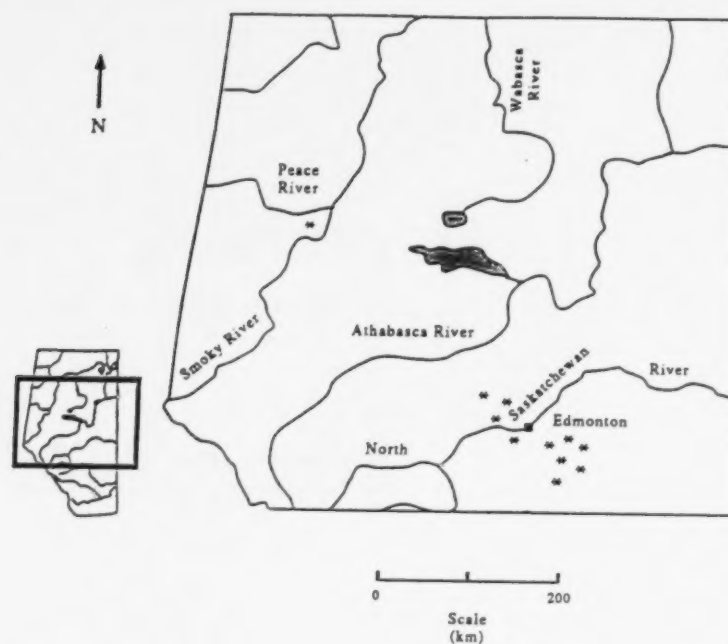


Figure 2. Map of Alberta, Canada with locations of the study lakes and farm dugouts sampled in 1990 indicated by an (*).

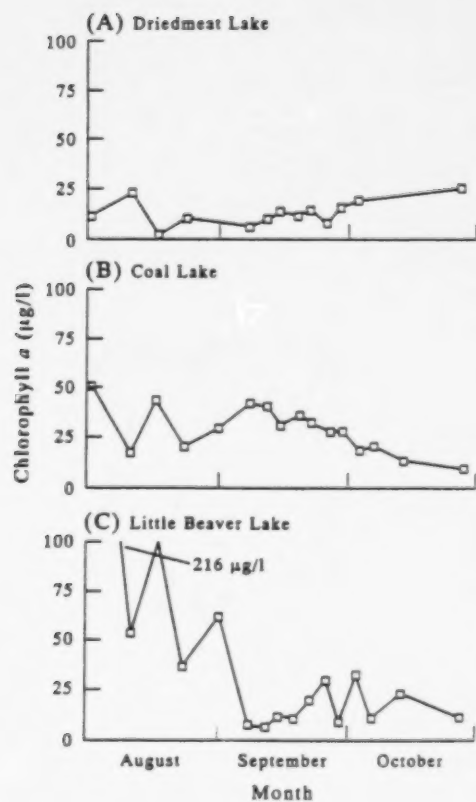


Figure 3. Surface (0 to 2.5 m for Driedmeat and Coal, 0 to 10 cm for Little Beaver) water chlorophyll *a* concentration in A) Driedmeat, B) Coal, and C) Little Beaver Lakes from August to October, 1990.

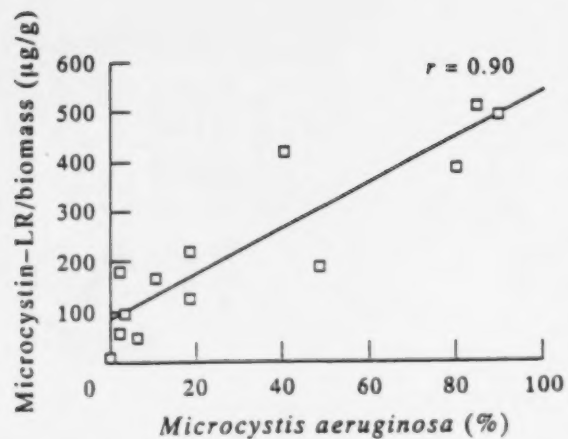


Figure 4. Relationship between relative abundance of *Microcystis aeruginosa* and the concentration of microcystin-LR in the bloom samples in Coal Lake, 1990.

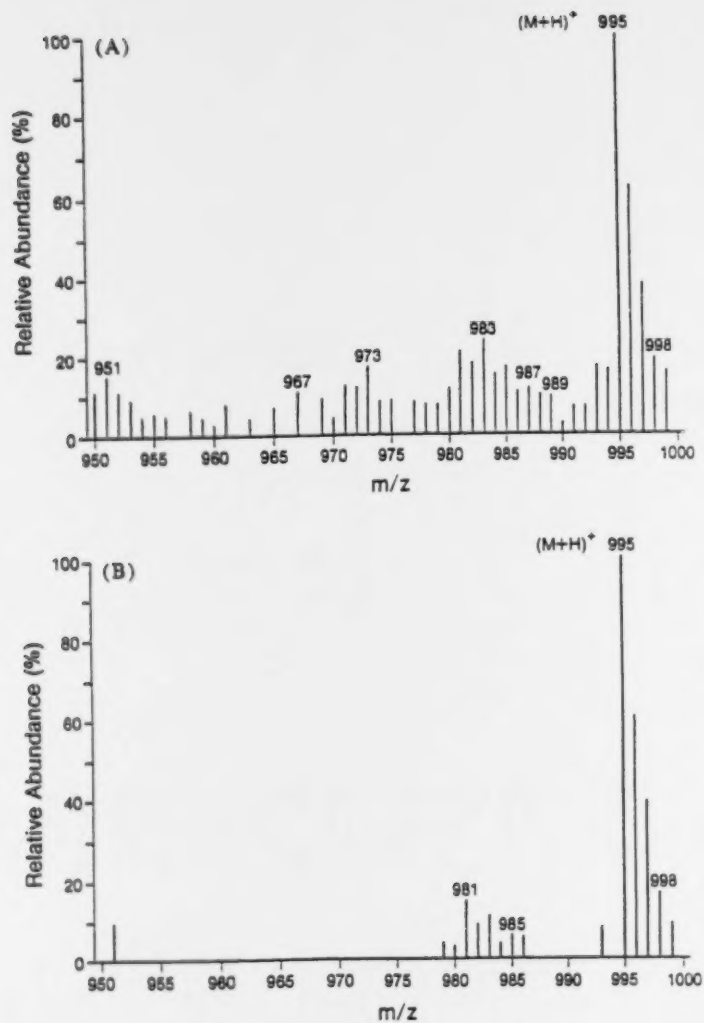


Figure 5. Pseudomolecular ion region of positive ion FAB spectra of A) toxin isolated from the combined bloom samples from Coal Lake, and B) purified microcystin-LR standard, in Magic Bullet Matrix.

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Chapter 4

Variability of microcystin-LR concentration in phytoplankton of hypereutrophic drinking water lakes²

Introduction

The production of neuro- and hepatotoxic compounds by freshwater cyanobacteria has been recognized by the scientific community since the late 1800s. These compounds have been implicated world-wide in poisonings of domestic livestock, pets and wildlife after the animals consume toxin-containing water and/or cyanobacterial cells from lakes, ponds and farm dugouts (Beasley et al. 1983; Edler et al. 1985; Soll and Williams 1985; Galey et al. 1986). Accidental ingestion of the toxins may also compromise human health (Dillenberg and Dehnelt 1960; Bourke et al. 1983; Falconer et al. 1983; Lawton and Codd 1991). Cyanobacteria known to produce hepatotoxins include species of *Microcystis* (Botes et al. 1985; Watanabe et al. 1986; Namikoshi et al. 1992a; Sivonen et al. 1992b; Kotak et al. 1993), *Anabaena* (Krishnamurthy et al. 1986; Harada et al. 1991; Namikoshi et al. 1992b, c; Sivonen et al. 1992a), *Nostoc* (Namikoshi et al. 1990; Sivonen et al. 1990a), *Oscillatoria* (Meriluoto et al. 1989; Bruno et al. 1992), and the brackish water *Nodularia spumigena* (Carmichael et al. 1988b; Sivonen et al. 1989).

With the exception of *Nodularia*, all other cyanobacteria named above produce cyclic heptapeptide hepatotoxins termed microcystins with the generalized structure cyclo(-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha-) where X and Z are variable L-amino acids, D-MeAsp is D-erythro-B-methylaspartic acid, Mdha is N-methyldehydroalanine and Adda is a novel amino acid (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Carmichael et al. 1988a). Lacking two amino acids of the microcystins, *Nodularia* produces a hepatotoxic pentapeptide, nodularin, with a structure

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very similar to the microcystins. Over fifty microcystins have been identified to date, most representing only minor components of the cyanobacteria from which they were isolated. *Microcystis aeruginosa* is capable of producing many microcystins simultaneously (Watanabe et al. 1989; Shirai et al. 1991). This study focused on the heptapeptide microcystin-LR (MC-LR) in three hypereutrophic lakes in Alberta, Canada. MC-LR is the dominant microcystin produced by cyanobacteria in these lakes (Boland et al. 1993). MC-LR is one of the most potent of the microcystins, having an LD₅₀ of 50 µg kg⁻¹ by intraperitoneal (i.p.) injection in mice; thus, it is more toxic to mice by i.p. injection than the organic contaminant and hepatotoxin 2,3,7,8-tetrachlorodibenzodioxin (2,3,7,8-TCDD) (World Health Organization 1989).

Adequate prediction of the occurrence of dangerous concentrations of toxic cyanobacteria has been hampered by a lack of information on the natural variability of the production, accumulation and degradation of toxins and relationship to environmental parameters. Risk assessment is difficult because, unlike point discharges of some anthropogenic contaminants, microcystins represent *in situ*, diffuse (non-point) pollution that may be produced within a water body continuously and variably. A few studies have attempted to monitor *in situ* variation of microcystin by analytical methods of detection such as HPLC (Lindholm et al. 1989; Watanabe et al. 1989, 1992; Wicks and Theil 1990; Shirai et al. 1991). However, the majority of studies examining cyanobacterial toxin variability in lakes have relied heavily on detection of toxins by mouse bioassay, a useful method as an initial toxicity screen, but which has a much higher detection limit than HPLC. The mouse bioassay method can detect toxin levels that may cause acute animal poisonings but is not sensitive enough to detect concentrations that may represent a chronic health risk. The method also cannot identify which of the many microcystin variants may be present in the cyanobacterial blooms. Little is known about the natural spatial or temporal variability of specific microcystins, such as MC-LR, in lakes, ponds and streams (Kotak et al. 1993) and how this *in-situ* variability relates to environmental factors.

The objectives of this study were: 1) to examine the variability of MC-LR in plankton from three lakes both temporally (changes within a year, between years and over

24 h) and spatially (differences between lakes on the same day and variation within a lake on a particular day) based on HPLC detection and quantitation of MC-LR, and 2) to correlate this variability to limnologically-important environmental variables (e.g. nutrients, water temperature, water clarity) and phytoplankton community composition. This study provides a long-term (i.e., 3 yr.) data base of the concentration of MC-LR, expressed in three units of measurement, in three lakes which serve as drinking water supplies and correlates toxin concentrations to limnological variables.

Materials and Methods

Temporal study-within and between year variation - The study lakes are hypereutrophic and serve as municipal drinking water supplies: Coal Lake for the City of Wetaskiwin (population 10,000), Driedmeat Lake for the City of Camrose (population 15,000) and Little Beaver Lake for the Village of Ferintosh (population 200). Water and plankton samples were collected from 1 August to 24 October 1990, 1 June to 11 October 1991, and 14 May to 30 September 1992 weekly or every two weeks from one central site (in 1990) or from four sites (in 1991 and 1992) in each lake. Data for 1991 and 1992 are reported as averages of the four sites.

Tygon tubing fitted with a one-way valve was used to collect water for chemical analysis and phytoplankton species composition. Samples were collected from the surface to a depth of 2 m from Coal and Driedmeat lakes in all three years. Due to the shallowness of Little Beaver Lake, (less than 2 m) water samples were either collected as surface water grabs (in 1990) or as integrated samples from the surface to a depth of 1 m (in 1991 and 1992). Water was analyzed for total phosphorus (TP) and total dissolved phosphorus (TDP) by the persulfate digestion method of Menzel and Corwin (1965) as modified by Prepas and Rigler (1982), although large particles ($> 250 \mu\text{m}$) were not filtered out. Forms of inorganic nitrogen monitored included nitrate and nitrite (referred to as nitrate (NO_3^-) in this paper, as nitrite concentrations are commonly non-detectable in the top 2 m of these lakes [$<0.5 \mu\text{g L}^{-1}$]), and ammonium (NH_4^+), analyzed by colorimetric methods on a Technicon AutoAnalyzer II (Stainton et al. 1977). Although ammonium was monitored in all years (1990-1992) in the three lakes, nitrate was only monitored in

1990 and 1991. Phytoplankton biomass was estimated as chlorophyll *a* after ethanol extraction (Bergmann and Peters 1980). Mean water column temperature and Secchi disk depth were also measured. Phytoplankton samples were preserved in Lugol's solution from surface water samples (1990 and 1991) or from integrated water samples in 1992 (0 to 2 m for Coal and Driedmeat lakes, 0 to 1 m for Little Beaver Lake). Phytoplankton were identified (as described by Prescott 1978) and enumerated with an inverted microscope equipped with a calibrated micrometer. Enumerations were made by filling a counting cell three times and evaluating 20 fields in each cell (i.e., 60 fields in total for each sample). The cyanobacteria were expressed as either a % of the total cyanobacterial volume (all data) or in terms of cell abundance (cells mL⁻¹) and volume (1991 and 1992 data).

To collect cyanobacteria for toxin analysis, a 64- μ m plankton net (29-cm aperture, 90-cm long, fitted with a 4.5 x 13-cm brass bucket) was skimmed across the waters' surface (in 1990 and 1991). In 1992, the net was hauled vertically through the water column from a depth of 2 m (Coal and Driedmeat lakes) or 1 m (Little Beaver Lake) to the surface so that the depth of collection of water samples for chemical analyses would be the same as the depth for collection of plankton for toxin analyses. Samples were then transported to the laboratory and freeze-dried for subsequent toxin analysis. Samples were extracted with 5% acetic acid and analyzed by reverse phase HPLC (Kenefick et al. 1992). Briefly, microcystins in the acetic acid extract were partially purified and concentrated on preconditioned SepPak C-18 cartridges (Supelco Co.) and eluted in 100% methanol. HPLC analysis was carried out on a Waters 6000A HPLC with a Hewlett Packard (HP) 1040A diode array detector and HP85 data processor. Samples were run under isocratic (10 mM ammonium acetate: acetonitrile, 74:26 v/v) conditions with U.V. detection at 238 nm. Microcystin-LR (MC-LR) is reported as μ g g⁻¹ of total plankton biomass (>64 μ m), the current, accepted method for expression of toxin content for cyanobacterial toxins (Watanabe et al. 1989, 1992; Shirai et al. 1991) as well as ng MC-LR L⁻¹ and μ g g⁻¹ *Microcystis aeruginosa*. This planktonic material may have contained zooplankton in addition to phytoplankton. The detection limit for MC-LR by HPLC was 1 μ g g⁻¹ plankton biomass. Toxin data were converted from μ g MC-LR g⁻¹ of

total plankton biomass (dry weight) to ng MC-LR L⁻¹ of lake water as follows: 1) the mass of planktonic material (dry weight) per liter was calculated based on the relationship between chlorophyll *a* and phytoplankton dry weight (APHA et al. 1989) and 2) multiplying the mass estimate by MC-LR concentration (µg g⁻¹ plankton biomass) to give µg MC-LR L⁻¹. The value was then re-expressed as ng MC-LR L⁻¹. To examine the validity of using the chlorophyll *a*-phytoplankton dry weight relationship (APHA et al. 1989) for determining the mass of phytoplankton in mixed plankton samples, I also determined total plankton mass for the 1992 data based on estimating the volume of water sampled with the phytoplankton net (assuming the net sampled a cylinder of water with 100% efficiency) and determining the mass of plankton collected after freeze drying. As sampling efficiency of plankton nets is variable and often much less than 100% (see Prepas and Vickery 1984) the mass of plankton determined by this method of calculation are likely underestimates. The mass of plankton calculated by the chlorophyll *a*-algal dry weight relationship was reasonably close to that determined by actually measuring the dry weight of material collected in the phytoplankton net ($r^2=0.81$, $P<0.0001$, $df=1,119$). I therefore chose to use the chlorophyll *a*-dry weight relationship to express all (1990-92) MC-LR data on a volumetric basis. Combining the toxin data (as ng MC-LR L⁻¹) and phytoplankton counts also allowed me to express MC-LR on a µg cell⁻¹ of *M. aeruginosa* basis. These calculations were done on the 1991 and 1992 data only and assumed that all of the MC-LR was produced by *M. aeruginosa* (see Kotak et al. 1993, and the discussion in this chapter). Data were then re-expressed as µg MC-LR g⁻¹ of *M. aeruginosa* by multiplying by a conversion factor of 32 pg (dry weight) cell⁻¹ (Reynolds 1984) and scaling the quantity to a per gram basis.

Spatial variation-within lakes - Spatial variability in MC-LR was studied on 1 and 14 August 1991 in Coal and Little Beaver lakes, respectively. Water samples were collected from a depth of 0 to 1 m with Tygon tubing fitted with a one-way valve every 300 to 350 m in Coal Lake and every 75 m in Little Beaver Lake, for the entire length of the lakes. Forty-five sites were sampled in Coal Lake and 29 sites in Little Beaver Lake. One water sample was collected per site and analyzed for chlorophyll *a* and phytoplankton community composition. Plankton samples for toxin analysis were collected by 0 to 1 m

vertical hauls of a 64- μ m plankton net, frozen and processed as described above. Secchi disk depth was measured at each site. To account for variability in parameters within a site, five replicate samples were collected for chlorophyll *a* and MC-LR at one site near the water treatment plant intake on Little Beaver Lake. MC-LR was expressed as μ g g⁻¹ of total plankton biomass, ng L⁻¹ of lake water and μ g g⁻¹ of *M. aeruginosa*.

Diel variation - Diel variation in MC-LR was studied in Driedmeat Lake from 1200 h on 9 September to 1200 h on 10 September 1992. Duplicate samples were collected from one central site (max. water column depth 3 m) every 3 h. Water samples were collected at 1-, 2- and 3-m depths. Depth profiles of water temperature, dissolved oxygen, pH and chlorophyll *a* were constructed from water samples collected at the 1-m depth intervals. Water samples were collected with a 1.5-L aluminum drop-sleeve water sampler, dissolved oxygen and water temperature were measured *in situ* with a YSI model 51 O₂/temperature meter, and pH was measured with a Fisher model 3D field pH meter. Secchi disk depth was measured throughout the daylight hours (approx. 0800 to 2100 h). Plankton samples for toxin analysis were collected in duplicate from a water depth of 2 m to the surface with a 64- μ m plankton net. These samples were frozen on dry ice until arrival at the laboratory, processed and analyzed for MC-LR as described above. MC-LR was expressed as μ g g⁻¹ of total plankton biomass, ng L⁻¹ of lake water and μ g g⁻¹ of *M. aeruginosa*, as described previously. MC-LR concentration is reported as the mean of duplicate samples $\pm$ SE. Phytoplankton abundance was enumerated from duplicate samples collected from a depth of 2 m to the surface of the water with Tygon tubing fitted with a one-way valve. Abundance (cells mL⁻¹) is reported as the mean of duplicate samples $\pm$ SE.

Statistics - The relationships between MC-LR and limnological parameters were examined by correlation analysis based on log-transformed monthly means (within year data) or untransformed data between sites (spatial studies). Multiple regression analysis was performed on log-transformed monthly means to determine the relationship between MC-LR and *M. aeruginosa* abundance and total phosphorus. Significant relationships were defined as $P < 0.05$.

Results

Within and between year variation - The general limnology of the lakes during 1990 and historically has been described in detail elsewhere (Mitchell and Prepas 1990, Kotak et al. 1993). In summary, all three lakes are shallow (maximum depth 2 to 5 m) and are well mixed by the wind during the ice-free season. The lakes have hard water (179 to 341 mg L⁻¹ alkalinity as CaCO₃) and range in salinity from fresh (conductivity 491 and 551 μ S cm⁻¹, Coal and Driedmeat lakes, respectively) to moderately saline (1223 μ S cm⁻¹, Little Beaver Lake). The three lakes lie within the same ecoregion and are located within 25 km of each other. Coal Lake was the least hypereutrophic of the three lakes from 1990 to 92 with average total phosphorus (TP) concentration in the surface waters (i.e. 0 to 2 m) of 119 μ g L⁻¹ during the summer (June to August) while average TP concentrations in Driedmeat and Little Beaver lakes were 289 and 259 μ g L⁻¹, respectively. TP concentrations tended to increase throughout the summer in each lake with higher concentrations recorded in late August. Chlorophyll *a* in the surface water varied substantially within and between years for each lake. While chlorophyll *a* generally decreased from 1990 to 1992 in Coal and Little Beaver lakes, concentrations in Driedmeat Lake increased greatly (Table 1). In 1992, large blooms of cyanobacteria persisted from early July to late August in Driedmeat Lake with chlorophyll *a* concentrations reaching 100 μ g L⁻¹ in the top 2 m of the water column. Floating scums of cyanobacteria caused chlorophyll *a* to exceed 150 μ g L⁻¹ on several occasions (data not shown). Seasonal (within-year) changes in chlorophyll *a* in Coal and Driedmeat lakes may have been driven in part by changes in TP (correlation between mean monthly TP and chlorophyll *a* based on log-transformed data pooled over the three years for Coal and Driedmeat lakes: $r=0.76$, $P<0.003$, $df=11$ and $r=0.62$, $P<0.03$, $df=11$, respectively) whereas within-year change in chlorophyll *a* in Little Beaver Lake was very weakly, if at all, related to changes in TP ($r=0.20$, $P>0.5$, $df=11$).

All three lakes contained the same general pool of cyanobacterial species but differed markedly in the patterns of seasonal and yearly dominance. The cyanobacteria common to all three lakes included *Microcystis aeruginosa*, *Aphanizomenon* (*Aph.*) *flos-aquae*, *Anabaena* spp. and *Gomphosphaeria* spp. (Figs. 1a-i). Cyanobacterial species

composition of the phytoplankton community in Coal Lake changed dramatically over the three-year study. In 1990 in Coal Lake, the dominant cyanobacteria were *M. aeruginosa* and *Gomphosphaeria* spp., and to a lesser extent, *Aph. flos-aquae* (Fig. 1a). *Microcystis aeruginosa* made up more than 80% of the cyanobacterial biomass, based on volume, by mid August and decreased quickly thereafter. In 1991 *Aph. flos-aquae* and *Gomphosphaeria* spp. were dominant (Fig. 1b), while *M. aeruginosa* was much less abundant than in the previous year. In 1992 the relative biomass of cyanobacteria as a whole decreased substantially, with cryptomonads and diatoms almost completely dominating the phytoplankton community (data not shown) from May through July. *Microcystis aeruginosa* was virtually absent from Coal Lake during 1992 (Fig. 1c), while the most abundant cyanobacteria were *Aph. flos-aquae* and *Gomphosphaeria* spp. The species succession of phytoplankton in Little Beaver Lake contrasted substantially with that of Coal Lake: during August and September in 1990 *Aph. flos-aquae* was the dominant phytoplankter (at times making up 100% of the phytoplankton community biomass; Fig. 1d), whereas *M. aeruginosa* was dominant in 1991 (Fig. 1e). As in the case for Coal Lake, diatoms and cryptomonads dominated the phytoplankton community in 1992 at the expense of the cyanobacteria (data not shown). In terms of cyanobacteria, *M. aeruginosa* was very abundant, at times making up more than 90% of the cyanobacterial community volume (Fig. 1f). The dominant phytoplankton in Driedmeat Lake followed yet another pattern: in 1990 *M. aeruginosa* was the dominant cyanobacterium, whereas in 1991 *M. aeruginosa*, *Aph. flos-aquae* and *Anabaena* spp. were dominant, and in 1992 *Aph. flos-aquae* and *Anabaena* spp. were dominant (Fig. 1g-i). Despite making up a smaller fraction of the phytoplankton volume in Driedmeat Lake in 1992 compared to 1991 (Fig. 1h, i), *M. aeruginosa* abundance (expressed as cells mL⁻¹) was almost 6-fold higher on average in 1992 as compared to 1991 (data not shown).

The concentration of microcystin-LR (MC-LR) in plankton varied substantially between years for each lake, regardless of units in which MC-LR was expressed (Fig. 2a-i). Expressed on a $\mu\text{g g}^{-1}$ of total plankton biomass basis, the highest concentrations of MC-LR in Coal Lake were recorded in 1990 (up to 605 $\mu\text{g g}^{-1}$; Fig. 2a). Concentrations were lower overall in 1991 (maximum MC-LR was 229 $\mu\text{g g}^{-1}$) and was non-detectable

(i.e. $<1 \mu\text{g g}^{-1}$) throughout 1992 (Fig. 2a). In contrast, the plankton in Little Beaver Lake contained little MC-LR in 1990 but had higher concentrations in 1991 (from 700 to 1552 $\mu\text{g g}^{-1}$; Fig. 2d) throughout August, September and October. In 1992, the maximum MC-LR concentration in Little Beaver Lake was recorded on 12 May (865 $\mu\text{g g}^{-1}$), with concentrations generally remaining below 400 $\mu\text{g g}^{-1}$ thereafter (Fig. 2d). MC-LR concentration in Driedmeat Lake followed yet a different pattern over the three years: MC-LR increased from a mean of 183 $\mu\text{g g}^{-1}$ of total plankton biomass in 1990 to 259 $\mu\text{g g}^{-1}$ in 1991 and 307 $\mu\text{g g}^{-1}$ in 1992 (Fig. 2g). The highest MC-LR concentration in Driedmeat Lake during the three year study was measured in plankton collected on 25 August 1992 (938 $\mu\text{g g}^{-1}$ of total plankton biomass).

In addition to yearly variation, there was also substantial within-year variation in the peak concentration of MC-LR in the plankton of the three lakes. In 1990, Coal Lake contained the highest MC-LR during mid August to the beginning of September, while MC-LR in Little Beaver and Driedmeat lakes peaked in late September (Fig. 2a, d, g). In 1991, maximum MC-LR was recorded in Driedmeat Lake at the beginning of July (no samples were obtained in June as there was insufficient biomass to attempt HPLC analysis on the plankton) and in late August in Coal Lake, while high concentrations of MC-LR were maintained in Little Beaver Lake from early August to mid October. Interestingly, the maximum MC-LR concentration in Little Beaver Lake during 1991 was measured on 9 October when water temperature was only 8 °C (Fig. 2d). In 1992, MC-LR was non-detectable in Coal Lake and the highest MC-LR concentration in Little Beaver Lake was measured on 12 May when water temperature was 10 °C (Fig. 2d). The highest MC-LR concentrations in Driedmeat Lake during 1992 were from samples collected from mid August to mid September. Thus, peak MC-LR concentrations were found anywhere from early spring to late fall and varied within lakes from year-to-year and between lakes in any one year.

MC-LR data were also expressed as ng L^{-1} . These data represent the average MC-LR present in the plankton per liter of water in the top 1m (Little Beaver Lake) or 2m (Coal and Driedmeat lakes) of the water column. The calculation represents a measure of the potential MC-LR available for release into the water, not a measure of MC-LR

actually dissolved in the water. Volumetric concentrations of MC-LR in Coal Lake were always low (Fig. 2b) in comparison with the other two lakes (Fig. 2e, h). MC-LR concentration in Little Beaver Lake increased greatly from 1990 to 1991, reaching concentrations in excess of $5,000 \text{ ng L}^{-1}$ in 1991 (Fig. 2e) and decreased in 1992. In Driedmeat Lake, MC-LR concentration increased from 1990 to 1992, reaching a maximum concentration of $2,988 \text{ ng L}^{-1}$ in August of 1992 (Fig. 2h). Maximum MC-LR concentrations, both on a $\mu\text{g g}^{-1}$ plankton biomass and ng L^{-1} basis, occurred when the abundance and biomass of *M. aeruginosa* was highest (data not shown).

In 1991 and 1992 I also examined the natural variability of MC-LR within *M. aeruginosa* which produces most, if not all MC-LR in the lakes studied (Fig. 2c, f, i). It is evident from these graphs that *M. aeruginosa* produced variable amounts of MC-LR within a year for each lake. The highest MC-LR concentration calculated in these study lakes was from a sample collected from Little Beaver Lake, on 3 July 1991 ($13,329 \mu\text{g g}^{-1}$ *M. aeruginosa*). Comparison of the MC-LR data for Little Beaver and Driedmeat lakes, expressed as either $\mu\text{g g}^{-1}$ plankton biomass or ng L^{-1} with data expressed as $\mu\text{g g}^{-1}$ *M. aeruginosa* showed that the maximum toxin concentration in the plankton or water (Fig. 2d, e, g, h) did not correspond to peak MC-LR production by *M. aeruginosa* (Fig. 2f, i). In summary, each of the lakes behaved independently in terms of the relative importance of cyanobacterial species and the concentration of MC-LR in plankton biomass, despite being located within the same eco- and climatic region. Peak MC-LR concentration varied within each year for a given lake and varied between the lakes.

Seasonal (within-year) patterns of MC-LR (expressed in the three different units) in the lakes were correlated with several limnological parameters. I found positive and highly significant correlations between MC-LR concentration and TP, TDP and chlorophyll *a* concentrations (Table 2) and negative correlations between MC-LR and nitrate concentration that were non-linear. There were no relationships between MC-LR and water temperature, regardless of how MC-LR concentrations were expressed (Table 2). The strongest correlations in the data set ($r = 0.61$ to 0.77) were between MC-LR and the abundance and biomass of *M. aeruginosa* (Table 2). Multiple regression analysis was performed to predict MC-LR concentration, as ng L^{-1} , with the two best correlates of MC-

LR: namely, mean monthly TP and the abundance of *M. aeruginosa* (cells mL⁻¹). The model explained 84% of the variance (i.e., $r^2=0.84$, $P<0.0001$, $df=2, 24$). The linear equation for predicting mean monthly MC-LR was

$$(1) \quad (\log)MC-LR = -5.14 + 1.32(\log)TP + 1.04(\log) M. aeruginosa \text{ abundance.}$$

Spatial Studies - Forty-five sites were sampled along the 15-km length of Coal Lake on 1 August 1991 to determine the spatial patterns of MC-LR within the lake. MC-LR was detected in the plankton only in 14 sites, and those were all located in the north end of the lake (Fig. 3a). Concentrations of MC-LR in the northern half of the lake were variable, ranging from non-detectable to 277 $\mu\text{g g}^{-1}$ of plankton biomass (non-detectable to 824 ng L^{-1}). No MC-LR was detected past Site 18 (close to the mid point of the lake). In contrast to the detectable concentrations of MC-LR found in 14 of 45 samples collected during the spatial study, no MC-LR was detected from a single sample from the lake, collected by my regular sampling method (collecting one sample from four sites in a lake and combining the samples prior to MC-LR analysis) on the same day.

In contrast to MC-LR, chlorophyll *a* was lowest in the north end of the lake up to Site 19 (25 to 45 $\mu\text{g L}^{-1}$) and increased sharply in the south end of the lake (52 to 104 $\mu\text{g L}^{-1}$; Fig. 3a). Correspondingly, water clarity was better in the north end of the lake (Secchi depth ranged from 1.0 to 1.5 m in the north end versus 0.6 to 1.0 m in the south end). Chlorophyll *a* and Secchi depth were highly correlated ($r = -0.83$, $P < 0.001$, $df = 43$).

The dominant phytoplankters based on mean cell volume in Coal Lake on 1 August 1991 were the diatom *Stephanodiscus* spp. (48%) and the cyanobacteria *Aph. flos-aquae* (31%), *Gomphosphaeria* spp. (10%) and *Lyngbya* spp. (7%), with *M. aeruginosa* making up only a small component of the phytoplankton community (3% of the total volume on average; Fig. 3b). *Microcystis aeruginosa* was found along the entire length of the lake; however, the highest biomass was recorded in the north end of the lake with cell volumes of 1.2×10^6 to $1.4 \times 10^5 \mu\text{m}^3 \text{ mL}^{-1}$ from Site 1 to 21 (Fig. 3b) and volumes below 79,000 $\mu\text{m}^3 \text{ mL}^{-1}$ beyond Site 21. The distribution of MC-LR roughly

paralleled that of *M. aeruginosa*. *Microcystis aeruginosa* contained variable concentrations of MC-LR along the transect of the lake, illustrated in the plot of site versus $\mu\text{g MC-LR g}^{-1}$ of *M. aeruginosa* (Fig. 3c). Expressed in these units, MC-LR ranged almost over three orders of magnitude from non-detectable ($<1 \mu\text{g g}^{-1}$) to $769 \mu\text{g g}^{-1}$ of *M. aeruginosa*, with consistently non-detectable MC-LR concentrations beyond Site 18.

At Little Beaver Lake MC-LR concentrations were quite variable between the 29 sites sampled (Fig. 4a) and were 7 times higher on average than in the sites with detectable MC-LR in Coal Lake. In contrast to the spatial study at Coal Lake, MC-LR was detected at all sites. Concentrations were highest in the south half of the lake and lowest in the north half of the lake (average MC-LR from Sites 1 to 15 was $1071 \mu\text{g g}^{-1}$ plankton biomass versus $596 \mu\text{g g}^{-1}$ from Sites 16-29). Expressed on a ng L^{-1} basis, MC-LR was highest at Site 1 with a concentration of 8185 ng L^{-1} (data not shown). On the same day, five replicate samples were taken at one site near the intake pipe of the water treatment plant (near Site 13) for the Village of Ferintosh on Little Beaver Lake to examine the within-site variability of MC-LR and chlorophyll *a*. Mean MC-LR concentration was $1357 \mu\text{g g}^{-1}$ of total plankton biomass with a coefficient of variation (CV) of 9%. Mean chlorophyll *a* was $63.5 \mu\text{g L}^{-1}$ with a CV of 2%. The CV for MC-LR and chlorophyll *a* in the entire lake was 36% and 48%, respectively, suggesting that the variation between sites was much greater than at one site. Chlorophyll *a* was higher in the south end of the lake than in the north end (mean chlorophyll *a* from Sites 1 to 16 was $58 \mu\text{g L}^{-1}$ versus $33 \mu\text{g L}^{-1}$ for Sites 16 to 29; Fig. 4a), thus contributing to its variability. Secchi depth mirrored the trend in chlorophyll *a*. The two parameters were highly correlated ($r = -0.69$, $P < 0.001$, $\text{df} = 27$).

The dominant phytoplankton, based on biomass (i.e. volume) in Little Beaver Lake on 14 August 1991 were cyanobacteria; a large proportion of *Aph. flos-aquae* (83% of the total phytoplankton community on average for the lake) and a smaller proportion of *M. aeruginosa* (15%; Fig. 4b). A very small fraction of the phytoplankton included the diatom *Cyclotella* sp. (0.2%) and the cyanobacteria *Phormidium* spp. (1.0%) and

Anabaena spp. (0.2%). In contrast to Coal Lake, high densities of *M. aeruginosa* were found along the entire length of Little Beaver Lake, reaching cell volumes in excess of $2.9 \times 10^6 \mu\text{m}^3 \text{mL}^{-1}$. *Microcystis aeruginosa* also contained variable concentrations of MC-LR (Fig. 4c). Concentrations of MC-LR g^{-1} of *M. aeruginosa* ranged from 449 to 8745 $\mu\text{g g}^{-1}$.

Diel variation - In September 1992, when diel variation of MC-LR was measured, water temperature was 10 to 11°C over the 24-h period and was uniform throughout the water column. Similarly, dissolved oxygen and pH remained constant at 7 to 8 mg L^{-1} and 9.5 to 9.6, respectively, over 24 h with no distinction between depths. Secchi depth was 50 to 70 cm during the daylight hours of the study.

Mean water column chlorophyll *a* decreased over the 24-h period from 21 $\mu\text{g L}^{-1}$ at 1200 h on 9 September to 15 $\mu\text{g L}^{-1}$ at 1200 h on 10 September. Chlorophyll *a* was well mixed (i.e. equal at the three depths sampled) during the daylight hours but tended to accumulate near the surface from 2100 to 0300 h ($23 \pm 6 \mu\text{g L}^{-1}$ at surface and $13 \pm 0 \mu\text{g L}^{-1}$ at depths of 1, 2 and 3 m by 0300 h; data not shown). The phytoplankton community was dominated by: *M. aeruginosa* (48% based on cell abundance), *Aph. flos-aquae* (38%), *Phormidium* spp. (12%) and *Cryptomonas* spp. (2%). The distribution of *M. aeruginosa* in the water column changed only slightly over the duration of the study. In phytoplankton samples collected over the same depth interval as that for the toxin analysis (i.e. 0 to 2 m), *M. aeruginosa* abundance decreased from a high of $8965 \pm 189 \text{ cells mL}^{-1}$ at 1200 h 9 September to 3814 ± 884 by 2100 h, and then increased, oscillating between 5722 and 7375 cells mL^{-1} for the next 15 h (Fig. 5a).

MC-LR concentration in the plankton changed over the 24-h study. MC-LR decreased from a high of $482 \pm 6 \mu\text{g g}^{-1}$ of plankton biomass at 1200 h 9 September to lower concentrations throughout the night ($65 \pm 17 \mu\text{g g}^{-1}$ of plankton biomass by 0300 h; Fig. 5b). When expressed on a $\mu\text{g g}^{-1}$ of *M. aeruginosa* basis, MC-LR was 2376 ± 72 on average from 1200 to 1800 h, and then concentrations decreased, reaching the lowest, $387 \pm 101 \mu\text{g g}^{-1}$ of *M. aeruginosa*, at 0300 h 10 September (Fig. 5c). MC-LR then increased to $1544 \pm 5 \mu\text{g g}^{-1}$ of *M. aeruginosa* by 0900 h 10 September. The changes in MC-LR

concentration (as $\mu\text{g g}^{-1}$ plankton biomass) reflected in part changes in the abundance of *M. aeruginosa* ($r=0.66$, $P=0.05$, $df=7$).

Discussion

Within and between year variation - Considerable differences were evident between the three lakes studied both within and between years, despite being located within the same climatic and ecoregion. Seasonal variation in the toxicity of cyanobacterial blooms within and between lakes has been recognized for many years. Monitoring of lakes for the presence of toxic blooms, however, has traditionally been based upon detection of the toxins by mouse bioassay (Schwimmer and Schwimmer 1968; Watanabe and Oishi 1980; Carmichael and Gorham 1981; Henning and Kohl 1981; Leeuwangh et al. 1983; Richard et al. 1983). The detection limit for cyanobacterial toxins by the mouse bioassay can be up to forty times higher than analytical techniques such as HPLC. For example, a 20 to 30g mouse can be injected with approximately 25 mg (dry weight) of algal material without causing secondary effects to the mice. The minimum microcystin concentration that can be detected (i.e. the minimum concentration causing the death of the mouse) is approximately $1 \mu\text{g}$ for MC-LR. Therefore the detection limit by mouse bioassay is about $40 \mu\text{g g}^{-1}$. The detection limit by HPLC in this study was $1 \mu\text{g g}^{-1}$. Despite a more widespread availability of laboratories now utilizing HPLC and its better detection limits, many monitoring studies still rely on mouse bioassays for detection of cyanobacterial toxins (Carmichael et al. 1988c; Lanaras et al. 1989; Repavich et al. 1990; Sivonen et al. 1989, 1990b), likely a result of the lower cost of analysis for mouse bioassays relative to HPLC.

Detectable concentrations of microcystin-LR (MC-LR) in the lakes were correlated strongly with the cyanobacterium *Microcystis aeruginosa*. Over the three years of this study, 226 plankton samples were collected during the seasonal, spatial and 24-h studies, 168 (74%) of which contained detectable ($> 1 \mu\text{g g}^{-1}$ of plankton biomass) concentrations of MC-LR. *Microcystis aeruginosa* was present in 97% of those samples containing detectable MC-LR, suggesting that *M. aeruginosa* was likely the producer of MC-LR in these lakes. The samples containing detectable MC-LR but no *M. aeruginosa*

(3% of the samples collected) had very low MC-LR, close to my detection limit, and it is likely that some *M. aeruginosa* cells were present in the phytoplankton samples but were not enumerated. *Anabaena* spp. is the only other cyanobacterium that was found in these lakes that also has been reported to produce MC-LR (Krishnamurthy et al. 1986; Harada et al. 1991; Namikoshi et al. 1992c; Sivonen et al. 1992a). However, *Anabaena* spp. was not correlated to MC-LR ($r=0.08$, $P>0.60$, $df=34$, based on log transformed monthly means), suggesting that *M. aeruginosa* was the main producer of MC-LR in these lakes.

Few field-based studies have addressed the question of which environmental variables may control toxin production in cyanobacteria. I examined the relationship between MC-LR concentration and several limnological variables (e.g. water chemistry, temperature and clarity, chlorophyll *a*, and the abundance and biomass of *M. aeruginosa*). Surprisingly, water temperature was not correlated to MC-LR concentration (Table 2), although a wide range of water temperatures were recorded (range: 7-24 °C). Many of the blooms in my study lakes reached peak toxicity at various times of the year when water temperatures were 10 to 15 °C. Thus, water temperature was not a good *in situ* predictor of MC-LR concentration.

MC-LR was positively correlated with the nutrient phosphorus (TP and TDP, Table 2) suggesting that progressive eutrophication (i.e. nutrient enrichment) of lakes by natural or anthropogenic activities (such as fertilizer runoff) may increase the quantity of MC-LR produced. MC-LR was also positively correlated with chlorophyll *a* ($r=0.25$ to 0.59), but this relationship would depend on the phytoplankton species present. The relationship between MC-LR and inorganic nitrogen (Fig. 6) appears to follow a negative exponential. Since *M. aeruginosa* does not possess heterocysts, it cannot fix atmospheric nitrogen in aerobic environments as do heterocystis genera such as *Anabaena* (Edler and Parker 1984). Many of the lakes located in central Alberta have very low concentrations of inorganic nitrogen relative to total phosphorus (E.E. Prepas unpublished data). The relationships between MC-LR and phosphorus and nitrogen in these study lakes suggests that MC-LR production in *M. aeruginosa* may be a response to a shift in the nitrogen to phosphorus (N:P) ratio caused by increased phosphorus input (from external or internal sources), but no corresponding increase in nitrogen input.

Wicks and Thiel (1990) also examined the variation in microcystin production (microcystin -YR, -LR, -FR, -YA, -LA, and -Laba) in the cyanobacteria of a eutrophic South African reservoir over 2.5 years, and attempted to relate the changes in toxin concentration to environmental variables. They found that toxin production was positively correlated to rates of primary production, solar radiation, water temperature, pH and % oxygen saturation of the water ($r_s = 0.52$ to 0.67) and was weakly, negatively correlated to chlorophyll *a* and soluble reactive phosphorus concentration ($r = -0.33$). Interestingly, they found a positive correlation between toxin concentration and water temperature which does not agree with my results. The range in water temperature in the South African study was smaller than in the present study (approx. 12-26 °C vs 7-24 °C in my study). Since my study lakes can be ice-covered for up to six months, I expected a strong relationship between water temperature and MC-LR concentration, a relationship which was not evident. The lack of significant correlations of microcystins and inorganic nutrients (nitrate, ammonium, soluble reactive phosphorus) found in the study by Wicks and Thiel (1990) are not surprising given that nutrient concentrations in Hartbeespoort Dam are usually in excess of phytoplankton requirements. Correlations were also based on small sample sizes ($n=16$ to 20).

Several laboratory studies have tried to elucidate the effects of various environmental factors such as light, temperature and nutrients on the production of cyanobacterial toxins. Many of these studies report conflicting results which may reflect differences in the strains of cyanobacteria. For example, studies by Gorham (1964), Runnegar et al. (1983) and Van der Westhuizen and Eloff (1985) demonstrate that temperature can have a marked effect on toxin production in *M. aeruginosa* in the 18 to 32 °C range, whereas Watanabe and Oishi (1985) and Ohtake et al. (1989) found temperature in this range had no or little effect on toxin production. Some laboratory studies have indicated that irradiance may have an effect on toxin production in *M. aeruginosa* (e.g. Watanabe and Oishi 1985; Van der Westhuizen and Eloff 1985; Utkilen and Gjølme 1992), and others have reported little change in toxicity when nutrients (P or N) are manipulated (Watanabe and Oishi 1985). However, one consistent pattern does emerge from these laboratory studies: the production of microcystins are tightly coupled

to the cell cycle of the organism (Van der Westhuizen and Eloff 1985; Watanabe and Oishi 1985; Ohtake et al. 1989; Kaya and Watanabe 1990), with maximum production in late log phase. It is possible that the large amount of spatial variation in MC-LR we found in Coal and Little Beaver lakes may reflect populations of *M. aeruginosa* which were in different stages of the cell cycle. Van der Westhuizen and Eloff (1985) also suggest that conditions that are optimal for growth may not be optimal for toxin production. This may be evident in the present study. Peak abundance (and hence peak growth) of *M. aeruginosa* generally occurred in August and September in my study lakes. However, maximum concentrations of MC-LR per gram of *M. aeruginosa* did not occur at these times (Fig. 2d-i). Although there was a statistical relationship between the abundance (cells mL⁻¹) or biomass ($\mu\text{m}^3 \text{ mL}^{-1}$) of *M. aeruginosa* and MC-LR (expressed as $\mu\text{g g}^{-1}$ of total plankton biomass), the correlation coefficients explained only 38-48% of the variation in MC-LR (i.e. $r=0.62$ to 0.69). The same can be said for expressing MC-LR as ng L⁻¹ or $\mu\text{g g}^{-1}$ *M. aeruginosa* (Table 2). The unexplained variation is likely due to a combination of environmental factors and differences in the growth phase of the cells. When MC-LR is expressed on a $\mu\text{g g}^{-1}$ of *M. aeruginosa* basis (Fig. 2c, f, i, 3c, 4c, 5c) it is clear that MC-LR concentration is variable within *M. aeruginosa* in field samples over time (hours, weeks, months) and space.

Carmichael and Gorham (1977, 1981) suggested that variation in the toxicity of blooms may be in part due to changes in the relative proportion of toxic and non-toxic strains of the toxin-producing species within the blooms. Evidence for this arises from field isolates of species from toxic blooms. Some isolates produce the toxins, and some appear to not produce toxins (Gorham et al. 1964; Shirai et al. 1991; Watanabe et al. 1991). Strain differences might explain part of the variability in concentrations of MC-LR in my study, as 70% of samples that contained no detectable MC-LR contained detectable ($>1,000 \text{ cells mL}^{-1}$) *M. aeruginosa*.

In 1992 I quantitatively sampled both cyanobacteria and zooplankton to allow me to account for some of the variation in MC-LR concentrations that I have observed in Driedmeat and Little Beaver lakes. For example, in 1992 zooplankton in Little Beaver Lake accounted for between 6 and 76% of the total mass of the sample that was collected

and subjected to HPLC analysis. The coefficient of variation of MC-LR in the total biomass (i.e. expressed as $\mu\text{g g}^{-1}$ of plankton biomass) over the sampling season of 1992 in Little Beaver Lake was 92%. Some of the variability in the $\mu\text{g g}^{-1}$ of plankton biomass values can be accounted for by the extra and variable mass of zooplankton in my samples. By accounting for the variable mass of zooplankton in each sample, the coefficient of variation only drops to 71%. Thus, even when zooplankton biomass is accounted for, there is still substantial variation in MC-LR within a year. However, the zooplankton may have also contained MC-LR as a result of feeding on the cyanobacteria (Watanabe et al. 1992). This variation in MC-LR is still evident when MC-LR is expressed as $\mu\text{g g}^{-1}$ of *M. aeruginosa* (Fig. 2c, f, l, 3c, 4c, 5c), avoiding the problems inherent with samples containing cyanobacteria and zooplankton which may contribute mass and MC-LR variably to the samples. The coefficient of variation in MC-LR in Little Beaver Lake during 1992 based on data expressed as $\mu\text{g g}^{-1}$ of *M. aeruginosa* was 255%. This raises a question as to which units MC-LR should be expressed in. In terms of assessing risk of poisoning to livestock, for example, MC-LR should be expressed volumetrically (ng L^{-1}). Few studies have reported microcystin concentrations in volumetric units however (Lindholm et al. 1989; Lindholm and Meriluoto 1991). The risk of a poisoning event for livestock will depend most greatly, among other factors, on the amount of water and cells ingested by the animal and also on the amount of MC-LR in the cells on a per litre basis, regardless of whether the toxin is dissolved in the water or bound within the cyanobacterial cells. Expressing MC-LR on a $\mu\text{g g}^{-1}$ of *M. aeruginosa* or plankton biomass may give insight into the effects of various environmental factors (e.g. light, nutrients) on MC-LR production at the cell or community level but gives little indication of the risk of poisoning in a lake.

Spatial variation - Substantial spatial variation in MC-LR concentration was found along the length of Coal and Little Beaver lakes during my two spatial studies in 1991. MC-LR, expressed as ng L^{-1} , was positively correlated to various measures of *M. aeruginosa* abundance and biomass (e.g. cells mL^{-1} , $\mu\text{m}^3 \text{mL}^{-1}$, and different ways of expressing these as percentages; Table 3) in Coal Lake. The high correlations with the Coal Lake data must be interpreted with some caution as many of the MC-LR levels were

non-detectable (see Fig. 3a) and therefore entered as 0 in the data set. This tended to put more emphasis on the 0,0 area of the scatter plots, increasing the correlation coefficients. In the Coal Lake spatial data set MC-LR (as ng L⁻¹) was negatively correlated to chlorophyll *a* and was not related to total phytoplankton abundance (Table 3). Chlorophyll *a* data for the Coal Lake spatial study indicate that areas of a lake with the most concentrated blooms (i.e. highest chlorophyll *a*) are not necessarily areas that have the greatest risk to, for example, pets or livestock. Part of the variation in MC-LR concentration was due to variability within *M. aeruginosa* (Fig. 3c), possibly as a consequence of differences in the mix of cell cycle phases between sites and/or specific toxicities of *M. aeruginosa* strains.

Carmichael and Gorham (1981) found substantial spatial variation in the toxicity of blooms, detected by mouse bioassay, in nearby Hastings Lake. They attributed this variation to differences in the species composition and in the relative proportion of toxic and non-toxic strains between the sites. Lindholm et al. (1989) and Lindholm and Meriluoto (1991) found vertical stratification of a microcystin produced by *Oscillatoria agardhii* in a drinking water supply. Most of the microcystin was located at a depth of 6 m, corresponding to the location of the metalimnion where the *Oscillatoria* tended to accumulate.

In the Little Beaver Lake spatial study, there was a positive relationship between *M. aeruginosa* and MC-LR, as in the Coal Lake study (Table 3). However, these relationships were weaker in Little Beaver Lake, perhaps owing to a greater variability in MC-LR and *M. aeruginosa* and the lack of polarity of the data in the scatter plots, as found in the Coal Lake data. There was no relationship between total cyanobacteria and MC-LR and a positive relationship between chlorophyll *a* and MC-LR (Table 3). The positive relationship of chlorophyll *a* and MC-LR in Little Beaver Lake and the negative relationship between the two parameters in Coal Lake may be due to the greater proportion of *Microcystis*-produced chlorophyll *a* in Little Beaver Lake, whereas most chlorophyll *a* in Coal Lake was from other groups of algae. The considerable variation in MC-LR concentration in the lake (Fig. 4a) was also evident when MC-LR was expressed

as $\mu\text{g g}^{-1}$ of *M. aeruginosa* (Fig. 4c) indicating large variability in MC-LR production within the species.

Diel variation - This is the first study that I am aware of that has documented the changes in MC-LR over a 24-h period in a lake. Variables such as temperature, dissolved oxygen and pH did not change appreciably over the study period. During the study, water temperatures were cool enough (10 to 11°C) to permit continual mixing whenever there was a light wind. Only slight vertical migration in *M. aeruginosa* (a species capable of producing gas vacuoles and therefore regulating its position in the water column) was detected, possibly due to the poor physiological condition of the cyanobacterium at this time of the year. MC-LR concentration (as $\mu\text{g g}^{-1}$ of plankton biomass) declined more than 7-fold over the night (Fig. 5b). Part of this decrease was caused by a large contribution to the mass of the sample by the amphipod *Gammarus* spp. at 0300 h. However, MC-LR concentrations did decrease 6-fold over the night when the data are expressed as $\mu\text{g g}^{-1}$ of *M. aeruginosa* (Fig. 5c): the decline in MC-LR occurred after sunset. Likewise, MC-LR increased in the morning when the sun rose (approximately 0700 h). MC-LR concentration or its production per cell may be coupled to photoperiod, since synthesis of the peptide likely requires ATP provided by photosynthesis. It remains unclear, however, why the content of MC-LR per mass of *M. aeruginosa* declined over the evening and night. MC-LR may be broken down and utilized within the cell during the night, or lost through leakage from the cells, or autolysis. These hypotheses requires further consideration.

Hepatotoxins in drinking water supplies - Microcystins present a complex problem when they occur in water bodies that are used for drinking water by livestock or humans. However, prediction of where and when toxic blooms will form is difficult. The enormous variability in the toxicity of blooms within and between years, and even within a lake on a particular day makes assessment of the potential risk to users a major concern. It appears that the variability of MC-LR is greatest between years within a lake and also seasonally within a lake (Table 4). For example, the average coefficient of variation for within year changes in MC-LR for the three lakes was 85% (Table 4). Assuming data from Coal Lake on 1 August 1991 were atypical, spatial variation of MC-LR was less than seasonal or

yearly variation, and diel variation may lie between within- and between-year variation (Table 4). The within-site variation of MC-LR was relatively low (CV of 9%). When an assessment of the precision of the HPLC determinations is considered it is obvious that most of the variability of MC-LR reported is due to natural variation and not analytical variability (Table 4), which was less than 4%.

Given the spatial variability of MC-LR suggested in this study, several samples must be collected to determine whether cyanobacterial toxins are present in drinking water supplies (especially those lakes for which many individual users draw water for human or livestock consumption) or lakes used for recreation (i.e. swimming). For example, my regular sampling design of the study lakes involved collecting a single sample from each of four locations in the lakes and then pooling the four samples prior to MC-LR analysis (in 1991 and 1992). On 1 August 1991, the day of the spatial study of Coal Lake, MC-LR was non-detectable in a sample collected by this regular monitoring method while MC-LR was detected in 14 of the 45 sites sampled during the spatial study on the same day (Fig. 2a, 3a). Sampling design should also account for short-term (e.g. diel) changes in toxicity due to changes in species and/or strain composition of the bloom, or changes in the production (or loss) of microcystin.

Given that more than fifty microcystins can be produced by cyanobacteria, it is important that techniques for monitoring all microcystins be integrated into monitoring programs focussed on human and livestock health. Although the dominant microcystin present in samples collected from Alberta lakes is MC-LR (Boland et al. 1993, Craig et al. 1993), other microcystins are present (Craig et al. 1993, Kotak unpublished). Based on the limited number of published studies, it appears that the dominant microcystin variants in bloom samples collected from various parts of the world may differ (e.g. MC-YR and -RR may dominate in Japan (Watanabe et al. 1989; Shirai et al. 1991), MC-LR, -FR, -YA, -YR and -LA in South Africa (Wicks and Thiel 1990) and MC-LR and -RR in the U.S.A. (Namikoshi et al. 1992a)). Craig et al (1993) suggest that microcystins more hydrophobic than MC-LR, even though present in lower concentrations in the blooms, may represent a disproportionate health risk. These hydrophobic microcystins may penetrate liver cells more easily than MC-LR or may have slower clearance rates. The availability of

microcystin standards lags greatly behind the identification of new microcystins, and a measure of the total microcystin pool is required. Biological methods of detection such as the protein phosphatase assay show promise to quantify total toxicity of samples (both bloom samples and raw and treated water) without the need for several microcystin standards (Lambert et al. 1994).

I found considerable differences between the three study lakes, with respect to MC-LR variation, despite the lakes being located within the same climatic and eco-region. The differences in MC-LR may be due to differences in the degree of eutrophy of the lakes. For example, Coal Lake was the least hypereutrophic (based on TP concentrations) during the 3-yr study, while Driedmeat Lake was the most hypereutrophic. Lakes within the same areas should therefore not be assumed to behave similarly in terms of both phytoplankton dynamics and MC-LR production. Each lake should be considered unique, particularly when it is a drinking water supply.

When expressed as ng L^{-1} (Fig. 2c, f, i), MC-LR concentrations observed during this study were not likely to cause livestock poisoning. The health risk to humans is not yet well characterized but water treatment processes which include FeCl_3 or KMnO_4 may release the toxin from the cyanobacteria during water treatment (Himberg et al. 1989). Copper sulfate has been shown to cause a substantial release of MC-LR from cyanobacterial blooms (Kenefick et al. 1993), and water treatment processes such as conventional flocculation, filtration and chlorination offer limited removal of microcystins from drinking water (Keijola et al. 1988; Falconer et al. 1989; Himberg et al. 1989). This was evident when elevated human plasma enzymes, indicative of hepatic injury, was clearly related to chemical treatment (copper sulfate) of a toxic bloom of *Microcystis* spp. in an Australian drinking water reservoir (Falconer et al. 1983). They surmised that the microcystins released into the water as a result of copper sulfate treatment, passed through the water treatment system and appeared in the general distribution system. An adjacent nearby population utilizing another reservoir for drinking water was unaffected.

My data represent integrated 0-to-2 m samples collected in the center of the lakes where intense blooms along the surface of the water rarely occur. Blooms accumulating along the shoreline (where livestock have access to the water) would likely have much

higher MC-LR concentrations per litre than at the center of the lake, thus markedly increasing the risk of poisoning. For example, cyanobacteria that had accumulated along the shore of Driedmeat Lake on 17 August 1993 was collected and analyzed for MC-LR. This material contained $742 \mu\text{g g}^{-1}$ MC-LR of plankton biomass, representing a concentration of approximately $6405 \mu\text{g MC-LR L}^{-1}$ (or 6.4 mg L^{-1}). Since, on average, cattle can consume 16 to 27 L of water per min (Smart 1991) it is conceivable that it could easily consume a lethal dose (Carmichael and Gorham 1977). This is one of the very few studies to date (Lindholm et al. 1989; Lindholm and Meriluoto 1991) which have expressed toxin concentrations on a volumetric (ng L^{-1}) basis. More attention needs to be directed to the ecology of toxic cyanobacteria and prevention or control. Based on the widespread occurrence of MC-LR in eutrophic drinking water supplies in Alberta (Kenefick et al. 1992, Kotak et al. 1993) and the liver tumor promoting capabilities of microcystins (Falconer 1991; Nishiwaki-Matsushima et al. 1992), Canada is developing water quality guidelines for MC-LR (Kuiper-Godman et al 1994).

Table 1. Mean summer total phosphorus (TP; $\mu\text{g L}^{-1}$) and chlorophyll *a* ($\mu\text{g L}^{-1}$) in the three Alberta lakes used as drinking water supplies. Samples were collected from the surface to a water depth of 2 m in Coal and Driedmeat lakes and either as surface grabs (1990) or surface to a water depth of 1 m (1991 and 1992) in Little Beaver Lake. Values represent mean concentrations in August for 1990 and for June to the end of August in 1991 and 1992. SE=standard error of the sample mean.

Lake	Year	TP		Chlorophyll <i>a</i>	
		Mean	SE	Mean	SE
Coal	1990	178	24	32	6
	1991	103	20	33	6
	1992	76	9	18	3
Driedmeat	1990	244	41	11	4
	1991	201	30	13	4
	1992	421	68	49	8
Little Beaver	1990	436	25	94	32
	1991	186	28	49	14
	1992	156	15	29	8

Table 2. Correlation analyses for MC-LR versus limnological parameters and phytoplankton community composition for data collected from all lakes. Correlation analyses were performed on log-log transformed data of monthly means. Analyses based on $\mu\text{g MC-LR g}^{-1}$ plankton biomass and ng MC-LR L^{-1} include data from 1990 to 92 and analysis based on $\mu\text{g MC-LR g}^{-1}$ *M. aeruginosa* include data from 1991 and 1992 only. df=degrees of freedom (i.e., n-2).

Parameter	$\mu\text{g MC-LR g}^{-1}$ Plankton Biomass			ng MC-LR L^{-1}			$\mu\text{g MC-LR g}^{-1}$ <i>M. aeruginosa</i>		
	r	P	df	r	P	df	r	P	df
<i>M. aeruginosa</i> (% of volume)	0.67	<0.0001	26	0.60	<0.0002	34	0.77	<0.0001	25
<i>M. aeruginosa</i> (cells mL^{-1})	0.69	<0.0001	26	0.72	<0.0001	26	0.61	<0.0009	25
<i>M. aeruginosa</i> ($\mu\text{m}^3 \text{ mL}^{-1}$)	0.62	<0.0001	34	0.64	<0.0003	26	0.61	<0.0009	25
TP ($\mu\text{g L}^{-1}$)	0.57	<0.0004	34	0.59	<0.0002	34	0.53	<0.005	25
TDP ($\mu\text{g L}^{-1}$)	0.49	<0.003	34	0.49	<0.003	34	0.46	<0.02	25
pH	0.47	<0.004	34	0.58	<0.0003	34	0.38	<0.05	25
Chlorophyll <i>a</i> ($\mu\text{g L}^{-1}$)	0.43	<0.009	34	0.59	<0.0002	34	0.25	>0.20	25
NO_3^- ($\mu\text{g L}^{-1}$)	-0.30	>0.15	19	-0.34	>0.12	19	-0.27	>0.35	11
Secchi depth (m)	-0.35	<0.04	34	-0.47	<0.004	34	-0.33	<0.10	25
Temperature ($^{\circ}\text{C}$)	0.02	>0.90	34	-0.001	>0.99	34	-0.13	>0.49	25
NH_4^+ ($\mu\text{g L}^{-1}$)	-0.06	>0.70	34	-0.10	>0.55	34	-0.08	>0.66	25

Table 3. Correlation analyses for MC-LR (ng L^{-1}) versus various phytoplankton parameters from Coal Lake, 1 August 1991 and Little Beaver Lake, 14 August 1991. Data were not transformed prior to analysis. Analyses for Coal and Little Beaver lakes are based on 43 and 27 degrees of freedom, respectively.

Parameter	Coal		Little Beaver	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Chlorophyll <i>a</i> ($\mu\text{g L}^{-1}$)	-0.52	<0.00003	0.87	<0.00001
Secchi depth (m)	0.44	<0.003	-0.56	<0.002
<i>M. aeruginosa</i>				
Density (cells mL^{-1})	0.76	<0.00001	0.33	<0.08
Volume ($\mu\text{m}^3 \text{ mL}^{-1}$)	0.76	<0.00001	0.33	<0.08
% of cyanobacteria density	0.94	<0.00001	0.61	<0.0004
% of cyanobacteria volume	0.93	<0.00001	0.63	<0.0003
Total cyanobacteria				
Density (cells mL^{-1})	-0.0008	>0.99	-0.13	>0.50
Volume ($\mu\text{m}^3 \text{ mL}^{-1}$)	0.07	>0.63	-0.38	<0.05

Table 4. Natural and analytical sources of variation of MC-LR ($\mu\text{g g}^{-1}$ plankton biomass) expressed in terms of the coefficient of variation (CV=standard deviation/sample mean $\times$ 100%).

Source of variation	Lake	Time Period	CV (%)	n
Between years	Coal	1990-92	111	3
	Driedmeat	1990-92	25	3
	Little Beaver	1990-92	94	3
Seasonal (within year)	Coal	1990	81	15
	Coal	1991	101	15
	Coal	1992	0	17
	Driedmeat	1990	68	6
	Driedmeat	1991	83	8
	Driedmeat	1992	105	21
	Little Beaver	1990	166	13
	Little Beaver	1991	65	13
	Little Beaver	1992	92	20
Spatial (between sites)	Coal	1 Aug 1991	185	45
	Little Beaver	14 Aug 1991	36	29
Spatial (within site)	Little Beaver	14 Aug 1991	9	5
Temporal (diel)	Driedmeat	9, 10 Sept 1992	49	9
Analytical-HPLC				
Replicate extractions			4	6
Retention time			4	10
Area counts			3	10

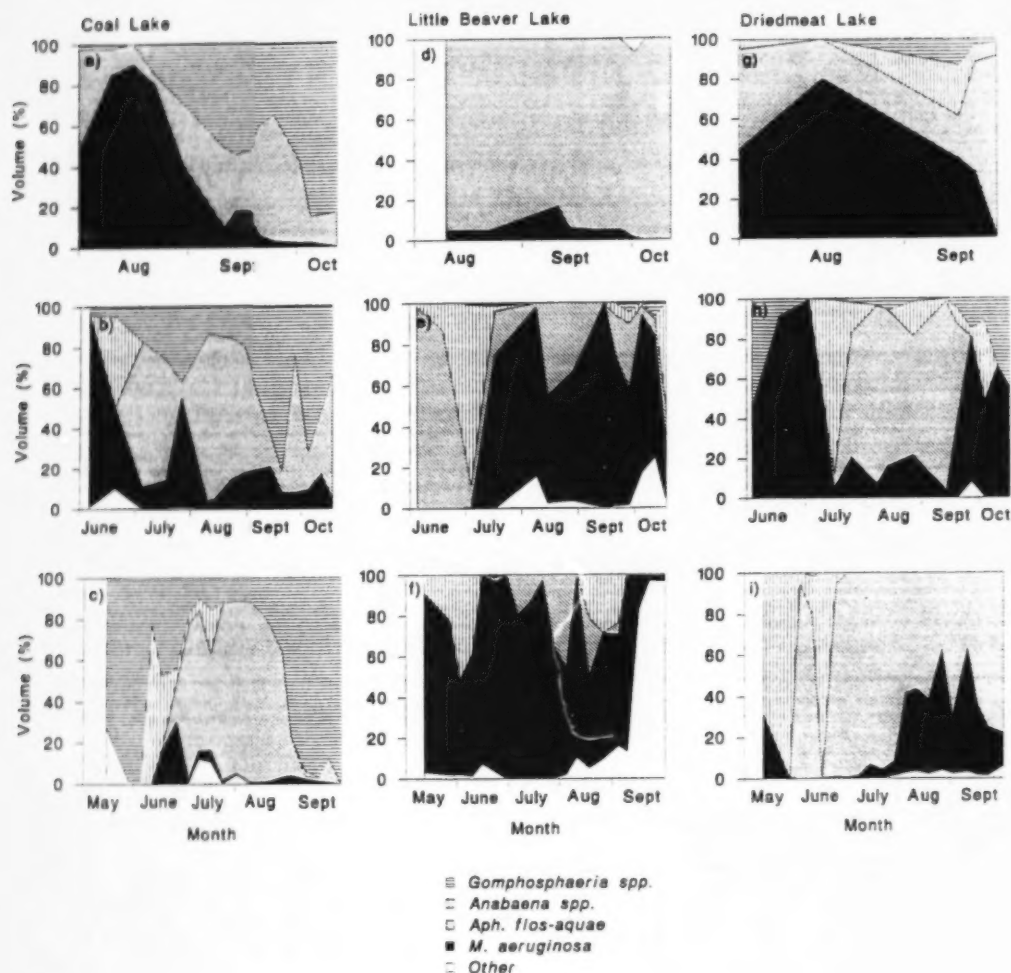


Figure 1. Seasonal changes in the cyanobacterial community composition in Coal Lake (left panel) a) 1990, b) 1991, and c) 1992, Little Beaver Lake (middle panel) d) 1990, e) 1991, and f) 1992, and Driedmeat Lake (right panel) g) 1990, h) 1991, and i) 1992. Samples from 1990 and 1991 were collected from the lake surface for all lakes, whereas 1992 samples were integrated water samples from the surface to a depth of 2 m in Coal and Driedmeat lakes, and the surface to a depth of 1 m in Little Beaver Lake. Community composition was expressed as a percentage of total cyanobacterial volume. "Other" category for Coal Lake includes *Phormidium* spp., *Lyngbya* spp. and *Merismopedium* spp., for Little Beaver Lake includes *Phormidium* spp., *Merismopedium* spp., *Oscillatoria* spp., *Chroococcus* spp. and *Coelosphaerium* spp. and for Driedmeat Lake includes *Phormidium* spp. and *Oscillatoria* spp.

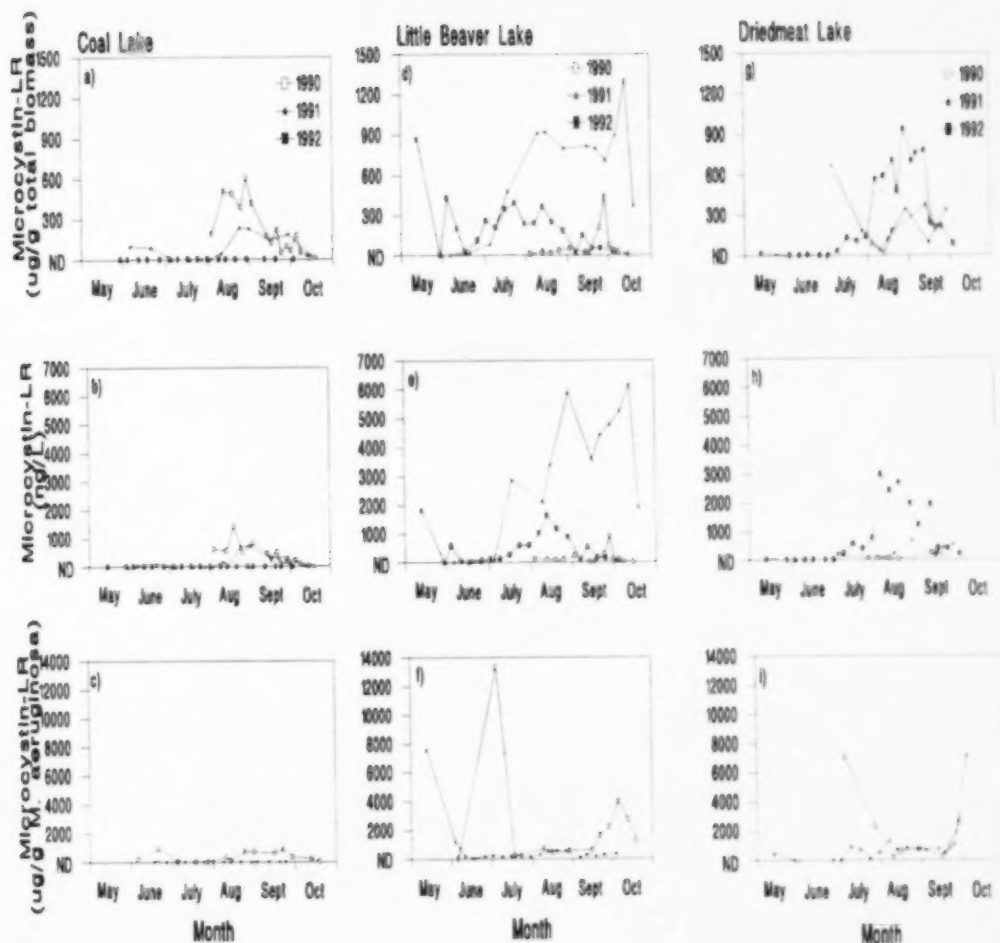


Figure 2. Concentration of microcystin-LR in Coal (left panel), Little Beaver (middle panel), and Driedmeat (right panel) lakes. Concentration of microcystin-LR is expressed as $\mu\text{g g}^{-1}$ of total plankton biomass for 1990-92 (top row), as ng L^{-1} for 1990-92 (middle row), and as $\mu\text{g g}^{-1}$ of *M. aeruginosa* for 1991 and 1992 (bottom row). Samples in 1990 and 1991 were collected from the lake surface, whereas samples collected in 1992 were from the 0 to 2 m stratum (Coal and Driedmeat lakes) or the 0 to 1 m stratum (Little Beaver Lake). ND = Not detected.

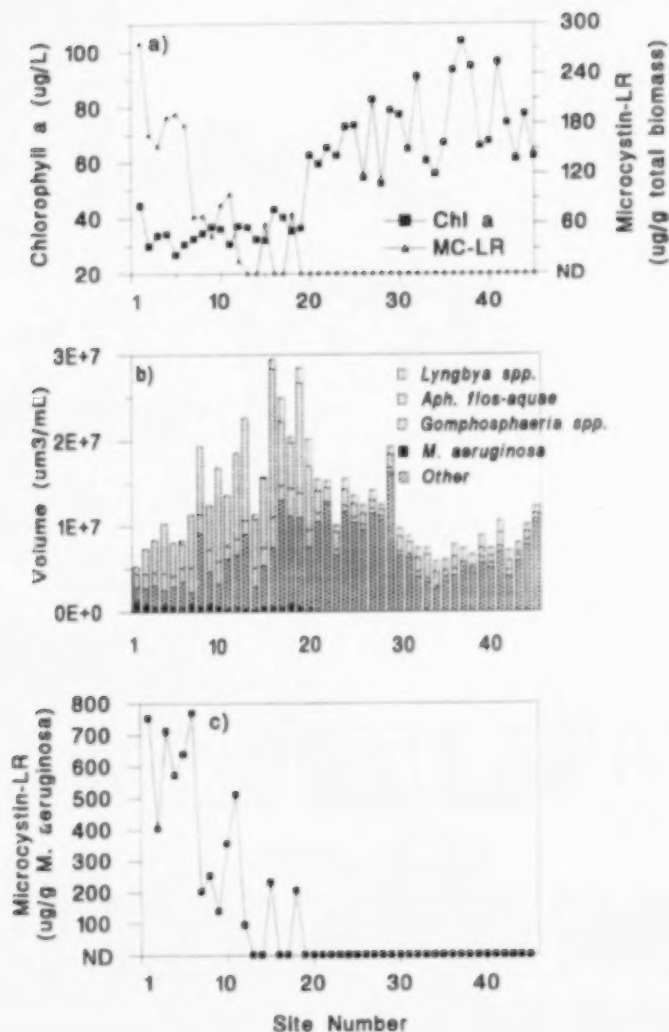


Figure 3. Spatial distribution of a) microcystin-LR concentration expressed as $\mu\text{g g}^{-1}$ of total plankton biomass and chlorophyll *a*, b) phytoplankton community composition based on cell volume in the 0 to 1 m stratum, and c) microcystin-LR concentration expressed as $\mu\text{g g}^{-1}$ of *M. aeruginosa* in Coal Lake, 1 August 1991. "Other" category in b) includes *Phormidium* spp., *Stephanodiscus* spp., *Anabaena* spp., *Ceratium* spp., *Pediastrum* spp. and *Asterionella* spp. ND = Not detected.

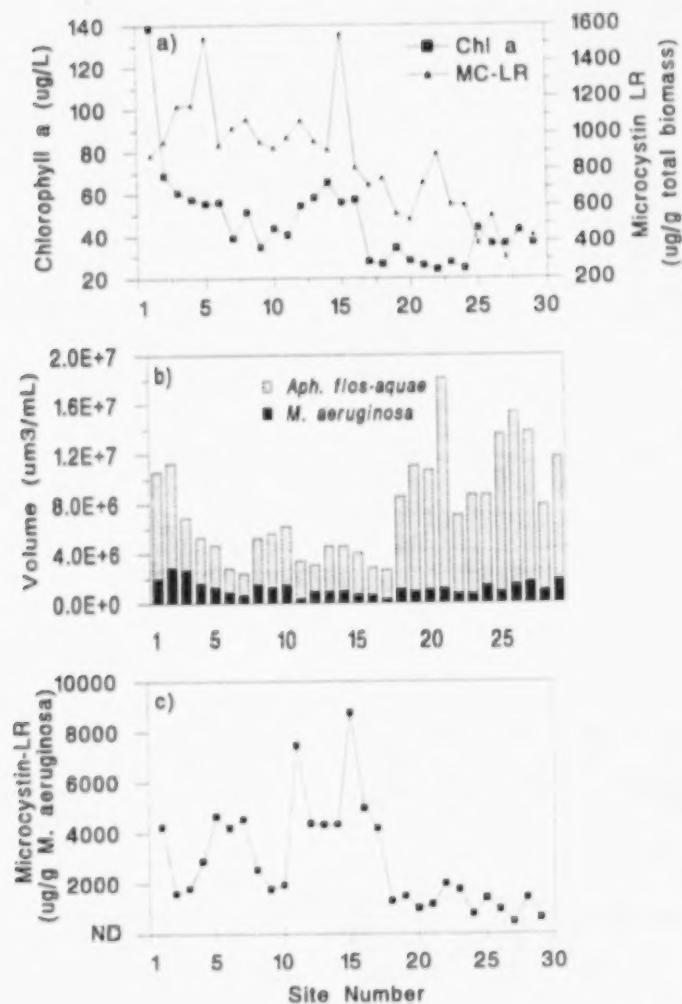


Figure 4. Spatial distribution of a) microcystin-LR concentration expressed as $\mu\text{g g}^{-1}$ total plankton biomass and chlorophyll *a*, b) phytoplankton community composition based on cell volume, in the 0 to 1 m stratum, and c) microcystin-LR concentration expressed as $\mu\text{g g}^{-1}$ of *M. aeruginosa* in Little Beaver Lake, 14 August 1991. ND = Not detected.

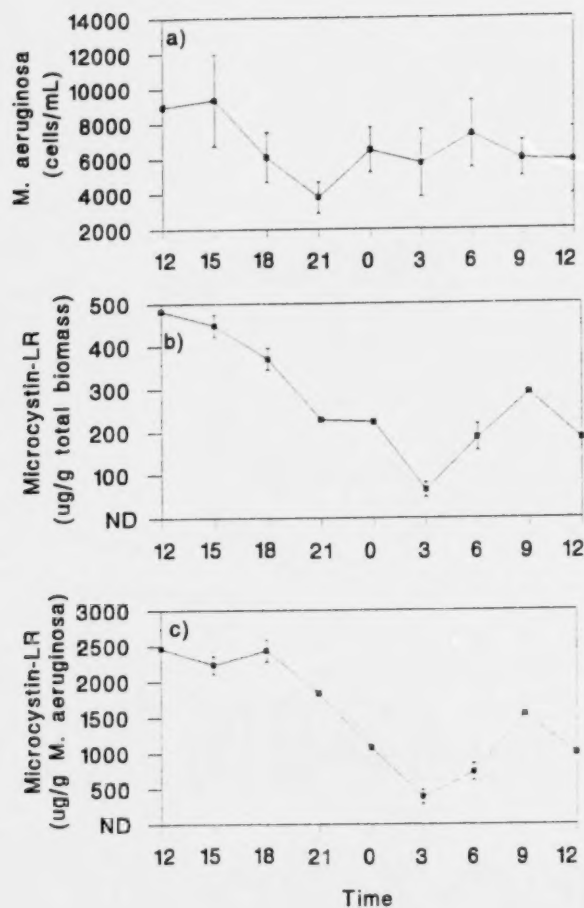


Figure 5. Average a) abundance of *M. aeruginosa*, b) microcystin-LR concentration expressed as $\mu\text{g g}^{-1}$ of total plankton biomass, and c) microcystin-LR concentration expressed as $\mu\text{g g}^{-1}$ of *M. aeruginosa* in the 0 to 2 m stratum of Driedmeat Lake on 9 and 10 September 1992. Vertical bars are the standard error of two replicate samples. ND = Not detected.

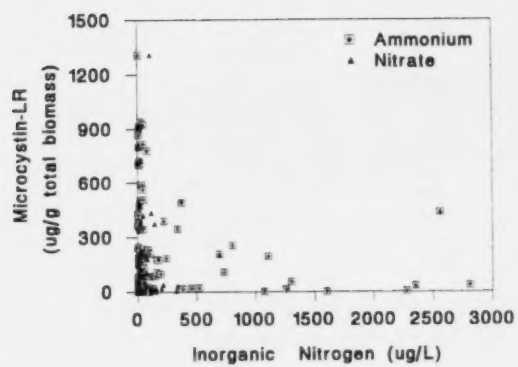


Figure 6. Relationship between microcystin-LR expressed as $\mu\text{g g}^{-1}$ of total plankton biomass and nitrate and ammonium in the three lakes.

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Chapter 5

Correlates and prediction of microcystin-LR concentration in phytoplankton from eutrophic lakes³

Introduction

Several genera of cyanobacteria produce potent liver toxins, termed microcystins, which have caused sporadic but repeated poisonings and deaths of animals worldwide (Schwimmer and Schwimmer 1968). Cyanobacteria that produce microcystins include species of *Microcystis* (Kotak et al. 1993, 1995), *Anabaena* (Harada et al. 1991), *Oscillatoria* (Meriluoto et al. 1989), and *Nostoc* (Namikoshi et al. 1990). Microcystins comprise a family of more than 50 closely-related cyclic heptapeptides (Carmichael 1994). Microcystin-LR (MC-LR) is the most commonly occurring analogue in central Alberta lakes (Boland et al. 1993; Craig et al. 1993; Kotak et al. 1993), one of the few regions worldwide where patterns of toxin concentration have been explored. MC-LR is also one of the most toxic microcystins, with an LD₅₀ of 50 µg kg⁻¹ in mice (Carmichael 1988). Microcystins are currently receiving widespread attention as a possible hazard to human health because of their ability to inhibit protein phosphatases in eukaryotic cells, which may lead to liver tumour promotion and an increased incidence of liver cancer (Fujiki and Suganuma 1993).

The reasons why cyanobacteria produce such compounds are unknown. Lampert (1981) suggested that microcystins may represent an evolutionary response to zooplankton grazing. Recently, this hypothesis has sparked numerous studies on the toxicity of microcystins and cyanobacterial neurotoxins to cladocerans, copepods and rotifers (e.g., Demott et al. 1991; Gilbert 1994; Reinikainen et al. 1994). Others have

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suggested that microcystin production may be a response to environmental stress (Lukac and Aegerter 1993; Kotak et al. 1995).

Several laboratory studies have examined the effects of various environmental factors (e.g., nutrients, light, temperature) on microcystin production in laboratory isolates of *M. aeruginosa* (e.g., Van der Westhuizen and Eloff 1985; Watanabe and Oishi 1985; Utkilen and Gjølme 1992). In these and similar studies only one variable is manipulated at a time while other variables are usually held at saturating conditions. Therefore the applicability to natural aquatic environments is questionable.

Because of their small size relative to most marine systems, lakes present an excellent opportunity to gain insights into the factors that may influence the production of cyanobacterial toxins in phytoplankton communities. In addition, the almost global occurrence of MC-LR in eutrophic to hypereutrophic lakes makes this particular microcystin analogue a logical choice for such studies. Only two research groups (Wicks and Thiel 1990; Kotak et al. 1995) have examined *in situ* microcystin concentrations of natural phytoplankton communities in detail. These studies have correlated toxin concentrations in samples of mixed phytoplankton assemblages to environmental variables including nutrients (nitrogen and phosphorus), water temperature and transparency, and phytoplankton species composition and biomass. However, the study by Wicks and Thiel (1990) was limited to one waterbody (Hartbeespoort Dam) in which nutrient concentrations were well above the requirements for phytoplankton, and because of its location (South Africa), the organisms may not be responding to the same factors as in north temperate lakes which can be ice-covered for 6 mo or more. While the study by Kotak et al. (1995) may be more representative of north temperate lakes, the study was restricted to only three lakes in central Alberta and only over a three-yr period. I also provide the only study where an attempt was made to quantify the relationships between MC-LR concentration in phytoplankton and environmental factors (Kotak et al. 1995), but my model was based on limited data. Given our limited knowledge on the patterns of MC-LR concentration, and other phytoplankton toxins, and how these patterns relate to changes in environmental variables, mathematical models based on a larger suite of lakes

and longer time series are urgently needed to adequately predict toxin occurrence and concentrations in lakes.

This study reports results of a survey conducted on up to 12 lakes (13 lake basins) in Alberta, Canada from 1990 to 1994 in which relationships between MC-LR concentration and environmental variables and cyanobacterial species were examined by correlation analysis. In addition, univariate and multiple regression models were developed to predict MC-LR concentration in the phytoplankton.

Materials and Methods

Study lakes - All of the thirteen lake basins were located in central and northern Alberta, spanning a distance of 510 km, and were within the aspen parkland and boreal-mixed wood ecoregions or boreal plains. The study lakes all have poor water quality (the lakes are eutrophic to hypereutrophic, see Mitchell and Prepas 1990), seven are used as drinking water supplies for cities and villages in Alberta or for livestock, all lakes except one are used extensively for recreational purposes (swimming, boating, fishing), and animal poisonings that were linked to cyanobacterial toxins have been reported in five of the lakes (O'Donoghue and Wilton 1951; Pybus et al. 1986; Mitchell and Prepas 1990; Kotak, personal observation). Two of the lakes in this survey (Figure Eight and Halfmoon S) have been treated on several occasions with lime (either as calcium hydroxide, calcium carbonate, or both) to reduce total phosphorus concentrations and algal biomass (Prepas et al. 1990; Babin et al. 1994). Baptiste Lake has two basins (N and S) and each basin was sampled separately. Eight of the 13 lake basins (Baptiste N, Baptiste S, Hastings, Isle, Lac la Nonne, Nakamun, Steele and Wizard) were sampled approximately every two weeks from 4 June to 4 September 1991, Figure Eight Lake was sampled six times (28 May to 8 October 1991), and the rest (Coal, Driedmeat, Halfmoon S and Little Beaver) were sampled approximately every two weeks from 4 June to 31 August 1991, and then roughly every week from the beginning of September to mid-to-late October 1991. In addition, six of the above lakes were also sampled in 1990, 1992, 1993 and 1994: Coal, Driedmeat and Little Beaver lakes (sampled from August to the end of October in 1990, May to the end of September in 1992, and May to the end of August in 1993 [September

1993-Driedmeat] and 1994), Figure Eight Lake (from May to the beginning of October in 1992), Halfmoon S Lake (from May to the end of October [1992] and September [1993]) and Hastings Lake (from May to the end of August in 1992).

With the exception of Little Beaver Lake, in which water was collected as surface grabs in 1990 and from the surface to a depth of 1 m in the remaining years, water samples in all other lakes were collected at four sites per lake with Tygon tubing fitted with a one-way valve (samples represent integrated water from the lake surface to a depth of 2 m). Water samples collected from the four sites were combined prior to chemical analysis. In 1991, water samples were analyzed for total phosphorus (TP), $\text{NO}_3^- + \text{NO}_2^-$, phytoplankton biomass (chlorophyll *a*; Chl_a), conductivity, pH and alkalinity on each sampling date. Other parameters (Cl^- , SO_4^{2-} , Na^+ , K^+ , Ca^{2+} , Mg^{2+} , color and turbidity) were analyzed on water samples collected every second sampling date in 1991. In the remaining years (i.e., 1990, 1992, 1993 and 1994), various limnological variables were dropped from our sampling program, and thus the decrease in the degrees of freedom for correlations based on the 1991 and the combined (1990-1994) data (Table 2).

TP was analyzed by colorimetric methods after potassium persulfate digestion (Menzel and Corwin 1965, as modified by Prepas and Rigler 1982), although large particles ($> 250 \mu\text{m}$) were not filtered out. $\text{NO}_3^- + \text{NO}_2^-$ and NH_4^+ were determined by colorimetric methods on a Technicon Autoanalyzer II (Stainton et al. 1977) and phytoplankton biomass was estimated as Chl_a, after ethanol extraction (Bergmann and Peters 1980). Cl^- and SO_4^{2-} were analyzed by ion chromatography on a Dionex Series 2000i/SP ion chromatograph, and Ca^{2+} , K^+ , Mg^{2+} and Na^+ by atomic absorption on a Perkin-Elmer Model 3300 Atomic Absorption unit, with methods described in APHA et al. (1992). In 1990 and 1991, total alkalinity was determined by manual titration of the water samples with 0.01 N sulfuric acid (APHA et al. 1992) and pH was measured on a Fisher Accumet Model 925 pH meter. In 1992 to 1994 total alkalinity was determined on an Mettler DL21 autotitrator. Conductivity, color and turbidity were measured on a Radiometer Analytical Model CDM83 conductance meter, a Hellige AquaTester Model 611-A meter, and a Hach Model 2100A turbidity meter, respectively. In 1993 total nitrogen was determined as the sum of total Kjeldahl nitrogen [D'Elia et al. 1977] plus

$\text{NO}_3^- + \text{NO}_2^-$. Water temperature was taken *in situ* at one central site in each lake with a YSI Model 51 temperature/dissolved oxygen meter from the surface to the sediments at 1-m intervals. Mean water temperature from the depth of water collection (e.g. 0 to 2 m or 0 to 1 m) was then calculated. Secchi depth was also recorded, at one central site. Phytoplankton were preserved in Lugol's solution from surface (0 to 0.5 m in 1990 and 1991, 0 to 2 m or 0 to 1 m in 1992 to 1994) water samples combined from the four sites for subsequent analyses. Phytoplankton were identified based on descriptions of Prescott (1978) and enumerated with an inverted microscope equipped with a calibrated micrometer (Kotak et al. 1995). Biovolume of each species was determined by estimating volumes from similar geometric shapes. Lake morphometry and watershed characteristics were obtained from Mitchell and Prepas (1990) and Kotak et al. (1993).

Microcystin-LR analysis - In 1990 and 1991 phytoplankton for microcystin-LR (MC-LR) analysis were concentrated from the lake surface (0 to 0.5 m) with a 64- μm plankton net. A minimum of five horizontal hauls were made per site. From 1992 to 1994 phytoplankton were collected by vertical hauls of the net over the same depth range for which water was collected for chemical analyses (0 to 2 or 0 to 1 m). Samples were de-watered (after combining the samples from the four sites) by passing them through a Plexiglass tube (15 cm height, 12 cm diameter) fitted with 64- μm Nitex netting on one end. The sample was then frozen immediately upon transport to the laboratory and later freeze-dried. Cells (50 to 100 mg) were extracted twice in 5% acetic acid, and microcystins concentrated and purified on disposable C18 cartridges (Supelco Inc., Bellefonte, PA., USA). Samples were then analyzed by reverse phase HPLC, as follows. From 1990 to 1992, MC-LR was quantified on a Waters 6000A HPLC with at HP 1040A diode array detector and HP85 data processor (Kenefick et al. 1992). Samples were run under isocratic conditions at a flow rate of 1 mL min^{-1} . The mobile phase consisted of 10 mM ammonium acetate and acetonitrile (74:26%). In 1993 and 1994 MC-LR was analyzed on a Waters LC Module I HPLC with a Waters model 746 data module. In both cases, MC-LR detection was based on UV absorbance at 238 nm. In 1993, samples were run under isocratic conditions at a flow rate of 1 mL min^{-1} . The mobile phase consisted of 10 mM ammonium acetate and acetonitrile (76:24%). In 1994, samples were run under

gradient conditions (20 to 30% acetonitrile in 10 mM ammonium acetate over 10 min, then 20% for the remainder of the run [30 min]) at a flow rate of 1 mL min⁻¹. A comparison of the two HPLCs and the three separation methods (two isocratic, one gradient) indicated no significant difference between the quantity of MC-LR detected, although the gradient method gave much better separation of MC-LR from other interfering peaks (Kotak 1995). MC-LR concentration, initially calculated as µg g⁻¹ of phytoplankton biomass, was then re-expressed volumetrically, as ng L⁻¹ (Kotak et al. 1995) and represented toxin concentrations within the cyanobacterial cells per L of lake water (0 to 2 or 0 to 1 m depth), not as dissolved (aqueous) toxin. To calculate MC-LR as ng L⁻¹, I first determined the biomass of phytoplankton in the water (dry weight L⁻¹) based on the published relationship between Chla and phytoplankton dry weight (APHA et al. 1992). MC-LR, as ng L⁻¹ was obtained by multiplying MC-LR as ng g⁻¹ by biomass (dry weight L⁻¹). MC-LR concentration was also expressed as µg g⁻¹ of *M. aeruginosa* biomass by combining the MC-LR data (as ng L⁻¹) and *M. aeruginosa* abundance (cells L⁻¹) and scaling the quantity of toxin to a per gram of *M. aeruginosa* basis. This calculation assumed that all MC-LR was produced by *M. aeruginosa* (see Kotak et al. 1993, 1995, discussion in this paper). The detection limit for MC-LR by both HPLCs and the three methods of analysis was approximately 0.5 to 1.0 µg g⁻¹ of plankton biomass.

In 1990 and 1991 the sampling depth for MC-LR and phytoplankton identification and enumeration was different from the depth of collection of water chemistry data in all years, and for phytoplankton data collected from 1992 to 1994 (0 to 2 or 0 to 1 m). Since all samples were collected in the middle of the lakes, where floating scums of cyanobacteria rarely occur, I assumed that phytoplankton species composition and MC-LR concentration would be similar between surface samples (0 to 0.5m) and integrated (0 to 2m and 0 to 1m) samples. Samples were collected for MC-LR and phytoplankton species composition in 1992 for both surface and integrated depths. MC-LR and phytoplankton biomass and species composition were similar between the two sampling methods (data not shown).

Statistics - To examine correlations between MC-LR concentration in phytoplankton (as ng L⁻¹) and as µg g⁻¹ *M. aeruginosa* and the biomass of *M. aeruginosa*,

limnological variables and cyanobacterial species composition data for individual sampling dates were log-transformed to linearize them and combined for all lakes. This data set (referred to as the combined data) represent all 13 lake basins sampled in 1991 and up to six of the lakes sampled for the entire 5-yr period. Similarly, the 1991 data set (representing all 13 lake basins in one yr) and the residual data set (combined minus 1991) were then analyzed by correlation analysis separately. Univariate linear regression models were constructed based on log-transformed or untransformed combined data to predict MC-LR concentration in phytoplankton (ng L^{-1}). In addition, stepwise multiple regression was used to predict MC-LR concentration in phytoplankton based on log-transformed combined data. Variables in the multiple regression were rejected from the model if their *P* value was >0.05 . SPSS for Windows statistical software (version 6.0) was used for all analyses.

Results

The study lakes - In 1991, all 13 lake basins were studied, and therefore descriptions of the general limnology and seasonal patterns of microcystin-LR (MC-LR) concentration in phytoplankton are made only for this year. All lakes were eutrophic to hypereutrophic based on mean summer total phosphorus (TP) and chlorophyll *a* (Chla) concentrations (Table 1). Lac la Nonne, Driedmeat and Little Beaver lakes had the highest mean summer TP concentrations (213, 201, and $186 \mu\text{g L}^{-1}$, respectively) while Baptiste Lake had the lowest mean summer TP concentration (35 to $57 \mu\text{g L}^{-1}$ in the two basins; Table 1). The highest mean summer Chla concentration ($79 \mu\text{g L}^{-1}$) in the upper 2 m of the water column was recorded in Lac la Nonne, which occasionally had surface (sampled from the lake surface) Chla concentrations exceeding $150 \mu\text{g L}^{-1}$. Massive fish kills also occurred in Lac la Nonne in August 1991 at a time when dissolved oxygen concentrations were less than 4 mg L^{-1} throughout the entire water column ($Z_{\text{max}} = 19.8 \text{ m}$) for more than 3 weeks. Mean summer Chla concentration in Nakamun and Isle lakes was also high (Table 1). Interestingly, Driedmeat Lake, which had one of the highest mean summer TP concentrations, had the lowest mean Chla concentration of all the lakes studied ($13 \mu\text{g L}^{-1}$). Subsequent to 1991 however, Chla concentrations in Driedmeat Lake

more than tripled (mean summer Chla concentrations for 1992, 1993 and 1994 were 49, 63 and 51 $\mu\text{g L}^{-1}$, respectively). The dominant ions in these hardwater lakes were: Na^+ , SO_4^{2-} and HCO_3^- (Table 1) with mean summer conductivity ranging from 249 to 846 $\mu\text{S cm}^{-1}$. Estimated water residence times ranged from 0.3 to >100 yr (Table 1). The lakes ranged from shallow to moderately deep (maximum depth from 2.5 to 27.5 m). Deeper lakes, such as Baptiste, Lac la Nonne and Wizard were thermally stratified by early summer, whereas shallower lakes (Little Beaver, Driedmeat, Coal, Figure Eight and Steele) were either weakly stratified or mixed throughout the sampling period. The lakes therefore represented a cross section of eutrophic, hardwater, and primarily freshwater lakes on the boreal plains.

Occurrence and concentration of microcystin-LR - A total of 90 phytoplankton samples were collected from the 13 lake basins in 1991 and analyzed by HPLC for microcystin-LR (MC-LR). The peak with a retention time corresponding to the MC-LR standard was almost always the largest peak in the HPLC chromatograms in all samples, suggesting that MC-LR was most often the major hepatotoxin in these samples. However, since I did not have standards for other microcystins, it is not possible to determine whether other microcystins were also present in the samples. Phytoplankton biomass was not sufficient to allow MC-LR analysis on several occasions in many of the lakes. Seventy percent of the samples collected (i.e., 63 out of 90) contained detectable concentrations of MC-LR. More than one-half of the lakes surveyed (Coal, Driedmeat, Halfmoon S, Hastings, Little Beaver, Nakamun, and Steele) contained detectable concentrations of MC-LR in more than 50% of the phytoplankton samples collected over the summer. In the remaining lakes MC-LR was detected <25% of the time (Figure Eight, Isle and Lac la Nonne) or only once (Baptiste N, S and Wizard) in the phytoplankton. All 13 lake basins had detectable concentrations of MC-LR at least once during 1991. In those lakes in which MC-LR was present in a high proportion of the samples collected over the summer, MC-LR concentration was generally highest during the latter part of the sampling period (Fig. 1b+c). The extreme was Little Beaver Lake where MC-LR was highest from late August to early October (Fig. 1c). The highest MC-LR concentration detected in 1991 (6174 ng L^{-1}) was from phytoplankton collected from Little Beaver Lake

on 9 October, when water temperature was only 8 °C. Lakes fell into three distinct categories based on MC-LR concentration averaged over the 1991 sampling period: low ($<50 \text{ ng L}^{-1}$; Baptiste N+S, Wizard and Figure Eight), intermediate (150 to $1,000 \text{ ng L}^{-1}$; Coal, Isle, Driedmeat, Lac la Nonne, Steele, Hastings and Halfmoon S), and high concentrations of MC-LR ($>1,500 \text{ ng L}^{-1}$; Nakamun and Little Beaver).

Relationship to environmental variables - MC-LR concentration in the phytoplankton (expressed as ng L^{-1}) in the combined data set was correlated with TP, Chla, Secchi depth, Na^+ , color, pH, turbidity and conductivity (Table 2). Many of the variables, such as Chla, pH and Secchi depth, however, are also autocorrelated. The strongest correlation between MC-LR and cyanobacterial biomass was with *Microcystis aeruginosa* ($r=0.52$, Table 2), one of the main producers of the toxin. There was no relationship between MC-LR concentration in the phytoplankton and *Anabaena flos-aquae*, the only other species that was present in the lakes that is capable of producing MC-LR (Table 2). MC-LR was also correlated to the biomass of *Phormidium* sp. ($r=0.52$, Table 2), likely a result of the association of *Phormidium* sp. with *M. aeruginosa* ($r=0.42$). *Phormidium* sp. is not known to produce MC-LR, and the biomass of this genus was always very low compared to *M. aeruginosa*. Results of the correlation analyses based on 1991 data alone were consistent with the analyses based on the combined data (Table 2). The direction of the trends were identical between the two data sets although correlation coefficients between MC-LR concentration and TP, *M. aeruginosa* biomass, Chla and Na^+ were slightly lower and those for conductivity, color, turbidity and Secchi depth were higher in the 1991 data set (Table 2). In either data set, there was no relationship between MC-LR in the phytoplankton and SO_4^{2-} , HCO_3^- and water temperature (Table 2) or any of the other ions (K^+ , Ca^{2+} , Mg^+ , Cl^- ; not shown). The relationships between MC-LR and $\text{NO}_3^- + \text{NO}_2^-$ and NH_4^+ in both data sets was not linear (Fig. 2a+b) and no transformation adequately linearized the data. Thus, the correlation coefficients for these relationships were not significant (Table 2), although there were clear negative exponential relationships between MC-LR and inorganic nitrogen (Fig. 2a+b). The outcome of correlation analyses of data from only six of the lakes sampled periodically from 1990 to 1994 (i.e., combined minus the 1991 data) were similar to the

other two data sets, although correlation coefficients for TP, Chla and Na^+ were much higher (Table 2). The correlation between *M. aeruginosa* and MC-LR was slightly higher in this data set than in the combined and 1991 data (Table 2) and the non-linear relationship between MC-LR and either $\text{NO}_3^- + \text{NO}_2^-$ or NH_4^+ was similar (not shown).

Because of the strong relationship between *M. aeruginosa* biomass and MC-LR concentration in the phytoplankton community, I hypothesized that MC-LR in phytoplankton might be influenced by factors that affect the biomass of *M. aeruginosa* produced or toxin production within *M. aeruginosa* cells, or both.

Since MC-LR in these lakes was likely produced predominantly or even exclusively by *M. aeruginosa*, I chose to also express MC-LR concentrations as $\mu\text{g g}^{-1}$ *M. aeruginosa* biomass. Thus I determined if the biomass of *M. aeruginosa* was correlated to the same limnological variables as those that affect MC-LR concentration in the species. Both *M. aeruginosa* biomass and MC-LR, expressed as $\mu\text{g g}^{-1}$ *M. aeruginosa*, were correlated with TP ($r=0.45$ and 0.46 , respectively), Chla ($r=0.49$ and 0.33), Secchi depth ($r=-0.36$ and 0.32) and pH in the combined data set ($r=0.34$ and 0.25 ; Table 3). However, whereas *M. aeruginosa* biomass was correlated to turbidity ($P=0.008$), MC-LR concentration per unit biomass was not ($P=0.15$) and MC-LR (as $\mu\text{g g}^{-1}$ *M. aeruginosa*) was correlated with conductivity, Na^+ and color ($P<0.05$) whereas *M. aeruginosa* biomass was not ($P>0.11$; Table 3). In addition, there were non-linear relationships between *M. aeruginosa* biomass and forms of inorganic nitrogen ($\text{NO}_2^- + \text{NO}_3^-$, NH_4^+), similar to those in Fig. 2a+b, but no such relationships were found between MC-LR concentration (as $\mu\text{g g}^{-1}$ *M. aeruginosa*) and inorganic nitrogen (data not shown). Therefore, *M. aeruginosa* biomass was not always correlated to the same limnological variables as MC-LR concentration per unit biomass of *M. aeruginosa*. MC-LR (as $\mu\text{g g}^{-1}$ *M. aeruginosa*) and *M. aeruginosa* biomass were also correlated ($r=0.46$; Table 3).

Separate linear regressions to predict MC-LR concentration in phytoplankton (as ng L^{-1}), based on the two variables that were most highly correlated with MC-LR in the combined data set (i.e., TP and *M. aeruginosa* biomass) were highly significant ($P<0.001$) but the proportion of explained variance was low ($r^2 < 0.30$ in both models; Table 4). However, the regression between MC-LR and TN:TP was significant ($P<0.02$) and a

larger proportion of the variance in MC-LR was explained by this variable ($r^2=0.70$; Table 4), although the original relationship between MC-LR and TN:TP was not linear (Fig. 3a). A stepwise linear regression model predicting MC-LR concentration in the phytoplankton initially included TP, *M. aeruginosa* biomass, Secchi depth, pH and conductivity. These variables had the highest *P* values in the correlations in the combined data set. During the stepwise analysis, conductivity and pH were removed from the model as their *P* values were well below the 0.05 level of significance and therefore MC-LR in the phytoplankton was predicted only from TP, *M. aeruginosa* biomass and Secchi depth. The equation was

$$(1) \quad \log \text{MC-LR (ng L}^{-1}) = -3.16 + 1.57 \log(\text{TP}) + \\ 0.25 \log(M. \text{ aeruginosa biomass}) - 0.92 \log(\text{Secchi depth}),$$

and explained 66% of the variation in the data ($r^2=0.66$, $P<0.001$, $df=3$, 206; Table 4).

Discussion

Microcystin-LR (MC-LR) concentration in phytoplankton in lakes can be highly dynamic in terms of within- and between-year, diel and spatial variation (Kotak et al. 1993, 1995, submitted). As much as 52% of the variability in seasonal changes in MC-LR in the phytoplankton from three hypereutrophic Alberta lakes (Coal, Driedmeat and Little Beaver lakes) was attributed to changes in the seasonal abundance and biomass of *Microcystis aeruginosa*, the main producer of MC-LR (Kotak et al. 1995). In the present study, MC-LR concentration in the phytoplankton was also strongly correlated to the biomass of *M. aeruginosa* ($r=0.52$, based on the combined data; Table 2). Despite the good correlation between MC-LR concentration and *M. aeruginosa* biomass and abundance in the present study, 73% of the variation (i.e., $1-r^2$) in MC-LR concentration in the phytoplankton was not explained by changes in the biomass of *M. aeruginosa*. I believe that environmental factors have an influence on toxin production within *M. aeruginosa* cells, as well as impacting the abundance of various strains of *M. aeruginosa* that produce no MC-LR (see discussion in Kotak et al. 1995). The presence in a critical

mass of the producer may set the potential for MC-LR production, and the environmental factors may modify the MC-LR production.

Given the importance of *M. aeruginosa* abundance and/or biomass in the total quantity of MC-LR in samples of mixed phytoplankton assemblages, it is important to understand the factors that regulate both the occurrence and biomass of *M. aeruginosa* in lakes, as well as toxin production within the *M. aeruginosa* cells. The factors which may regulate *M. aeruginosa* biomass/abundance in lakes need not be the same as those that regulate toxin production. To investigate this, I expressed MC-LR concentration on a per g basis of *M. aeruginosa* and compared the factors which were correlated with MC-LR with those correlated to *M. aeruginosa* biomass (Tables 2+3). Both the biomass of *M. aeruginosa* and MC-LR (as $\mu\text{g g}^{-1}$ *M. aeruginosa*) were strongly correlated with TP, Chla, Secchi and pH, suggesting that these variables may affect both *M. aeruginosa* biomass and toxin production per unit of *M. aeruginosa* biomass. However, turbidity, conductivity, Na^+ and color were correlated only with *M. aeruginosa* biomass or MC-LR, not both. Therefore, it is likely that certain limnological variables act directly on the growth (and hence the biomass produced) of *M. aeruginosa* and others on MC-LR production. Van der Westhuizen and Eloff (1985) examined microcystin production in laboratory strains of *M. aeruginosa*, and concluded that conditions that were optimal for growth (e.g., water temperature $>30^\circ\text{C}$) was not optimal for toxin production.

The production of microcystins by cyanobacteria may also be greatly affected by the physiological condition of the cells. Van der Westhuizen and Eloff (1985) found that microcystin production was tightly coupled to the cell cycle in laboratory *M. aeruginosa* strains, with maximum toxin production occurring in late log phase. I found a correlation between MC-LR concentration in *M. aeruginosa* and *M. aeruginosa* biomass ($r=0.46$). If I use biomass as an indicator of stage in the life cycle, then maximum MC-LR concentration in the present study also occurred during periods of maximum growth (i.e., late exponential growth phase).

The mechanisms by which environmental factors effect toxin production by cyanobacteria are poorly understood. Factors studied include water temperature, light intensity and quality, and micro and macro nutrients. Although nutrients have long been

suspected as a contributing factor to both cyanobacterial abundance and toxin production, surprisingly little information has been published on how, for example, changes in phosphorus and nitrogen, may affect microcystin production.

Phosphorus was the nutrient in this study which was most strongly correlated with MC-LR concentration in the phytoplankton. TP was a correlate of MC-LR concentration expressed on both a ng L^{-1} and $\mu\text{g g}^{-1}$ *M. aeruginosa* basis. In contrast, Watanabe and Oishi (1985) found little change in the toxicity of *M. aeruginosa* under culture conditions when phosphorus concentration in the media was reduced to 1/10 and 1/20 of the original culture concentration. However, the original concentration of phosphorus was incredibly high (100 mg L^{-1} of orthophosphate) compared to what is found in fresh water, and even at 1/20 of the original concentration, P was likely not limiting. Additionally, the actual microcystin content of the cells was not measured directly. Mouse bioassays were used as a correlate of microcystin content, a method which is less sensitive than analytical detection such as with high performance liquid chromatography (HPLC). For example, HPLC can be up to 40 times more sensitive than analyses by mouse bioassay for detecting MC-LR. In addition, the mouse bioassay method cannot distinguish between the more than 50 microcystin analogues, and the presence of microcystins can be completely masked if a cyanobacterial neurotoxin is present in the sample (Kotak et al. 1995). In contrast to the above study, Sivonen (1990) found that orthophosphate concentration had a marked effect on [D-Asp³]-microcystin-RR production in laboratory cultures of *Oscillatoria agardhii* at low P concentrations (100 to $400 \mu\text{g L}^{-1}$) but not at higher P concentrations. I have previously reported significant correlations between MC-LR concentration in phytoplankton and phosphorus (TP or TDP) in Coal, Driedmeat and Little Beaver lakes from 1990 to 92 (r up to 0.59; Kotak et al. 1995). Data from the present study also show strong correlations between MC-LR and TP (Tables 2+3).

There was a strong negative exponential relationship between MC-LR concentration and $\text{NO}_3^- + \text{NO}_2^-$ and NH_4^+ (Fig. 2a+b). I found a similar pattern when examining data from Coal, Driedmeat and Little Beaver lakes from 1990 to 1992 (Kotak et al. 1995). Despite the lack of linear association (Tables 2 + 3), it is evident that maximum MC-LR concentration occurred when sources of inorganic nitrogen were at

their seasonal lowest in the lakes ($<10 \mu\text{g L}^{-1}$ for $\text{NO}_3^- + \text{NO}_2^-$ and $<30 \mu\text{g L}^{-1}$ for NH_4^+ ; Fig. 2a+b). Rapala et al. (1993) examined the effect of nitrogen concentration and source (N_2 , NO_3^- , NH_4^+ and urea) on the production of the cyanobacterial neurotoxin, anatoxin-a, in several laboratory strains of *Aphanizomenon* and *Anabaena*. They found that toxin production was highest when cells were grown in N-free media and was lowest when grown in N-rich media. It should be noted however, that both genera are capable of N_2 -fixation in oxic environments whereas *Microcystis* is not. In contrast, Sivonen (1990) found that production of [D-Asp³]-microcystin-RR in *O. agardhii* increased with increasing nitrate concentration. The differences in the response of toxin production to N or P manipulation is not surprising since the response might be expected to be species-dependent. In addition the function of anatoxin-a and microcystins in the cyanobacteria may be very different, and therefore, the response to changes in N and P would be expected to differ.

The absolute concentration of total phosphorus and the ratio of total nitrogen to total phosphorus has been suggested to affect cyanobacterial abundance (Smith 1983, Trimbee and Prepas 1987). I measured total nitrogen (TN), TP, MC-LR concentration in phytoplankton and *M. aeruginosa* biomass in Coal, Driedmeat and Little Beaver lakes during the summer of 1993. MC-LR concentration in the phytoplankton was dramatically reduced when TN:TP was greater than 5:1 (Fig. 3a). Of all the regression models tested, MC-LR concentration in phytoplankton was best predicted from TN:TP. The impact of the TN:TP ratio on MC-LR concentration in the phytoplankton was manifested mainly by the affect of TN:TP on *M. aeruginosa* biomass (Fig. 3b), but not on MC-LR concentration in *M. aeruginosa* (Fig. 3c).

Light intensity has also been shown to have a marked effect on MC-LR production under culture conditions. Generally, microcystin production is highest at lower light intensities (Watanabe and Oishi 1985, Van der Westhuizen and Eloff 1985, Utkilen and Gjølme 1992). MC-LR concentration in the phytoplankton and turbidity and color were correlated in the present study (Table 2), suggesting that MC-LR production was higher under lower light conditions in lakes. The negative correlation between MC-LR in phytoplankton and Secchi depth in the present study ($r = -0.34$ to -0.43 ; Table 2) also

supports this hypothesis. However, closer examination of the data suggests that the main effect of turbidity on MC-LR concentration in the phytoplankton was by its affect on *M. aeruginosa* biomass and not on the concentration of the toxin in the *M. aeruginosa* cells (Tables 2+3), and that color may influence the concentration of MC-LR in the cells, but not *M. aeruginosa* biomass. In general, correlations drawn between environmental variables and parameters associated with gas-vacuolate species such as *M. aeruginosa* should be interpreted with caution, because these species are able to rapidly change their position in the water column (Reynolds 1987, Paerl 1988). The limnological conditions (light, nutrients, water temperature) that the colonies are exposed to will also change rapidly, particularly in deep stratified lakes (for example, in four of the 13 lakes basins in this study).

I also investigated the relationship between MC-LR concentration in the phytoplankton and several lake morphometric and watershed characteristics. MC-LR concentrations were averaged over the summer period in 1991 and correlated to morphometric and watershed characteristics. Mean summer MC-LR was negatively correlated to mean and maximum lake depth ($r = -0.50$ and -0.56 , respectively). This should not be surprising if *M. aeruginosa* abundance/biomass is an important factor controlling the concentration of MC-LR in the water column. Genera such as *Microcystis* tend to be more prominent in shallow lakes which are hydraulically unstable (i.e., lakes which can turn over rapidly due to wind-induced turbulence) (Reynolds 1987). I found, however, no relationship between MC-LR concentration and water residence time (range: 0.3 to >100 yr, $r = 0.13$, $P > 0.75$, $df = 9$). There was also no relationship with the ratio of watershed area to lake surface area (a surrogate for estimating potential external nutrient load). This was expected since internal P loading commonly make up a high proportion, if not almost all of the total P load to the euphotic zone on an annual basis in central Alberta lakes (Shaw and Prepas 1990).

Only two other studies have examined seasonal patterns of microcystin concentration in phytoplankton and related such patterns to environmental factors. A study of more limited scope (Kotak et al. 1995), evaluated MC-LR concentrations in three of the lakes examined in the present study (Coal, Driedmeat and Little Beaver) over

shorter time period (1990 to 1992). Correlations between MCLR concentrations in phytoplankton and variables such as *M. aeruginosa* biomass, Chla, TP, Secchi depth and pH, a similar negative exponential relationship between MC-LR and either $\text{NO}_3^- + \text{NO}_2^-$ or NH_4^+ , and no relationship between MC-LR and water temperature based on monthly means are consistent with the larger data set assembled in the present study. The lack of relationship between MC-LR and water temperature is intriguing since, although not explicitly stated in the literature, maximum cyanobacterial toxin concentration in phytoplankton is often assumed to occur during the summer when water temperatures are at their highest. We have recorded seasonal high toxin concentrations in Driedmeat and Little Beaver lakes in spring and autumn when water temperatures were below 10 °C (Kotak et al. 1995). The occurrence of neurotoxic blooms of *Anabaena flos-aquae* in ice-free American Lake, Washington, during the winter by Jacoby et al. (1994) support the premise that toxin-producing blooms are not restricted to warm summer months. Wicks and Thiel (1990) provide the only other study which has attempted to correlate microcystin concentrations in phytoplankton to environmental variables. They found that microcystin concentration (total of six microcystin analogues) in Hartbeespoort Dam, South Africa phytoplankton over 2.5 yr was positively correlated to biomass-normalized primary production, solar radiation, oxygen saturation of the water, water temperature and pH, and was negatively correlated to Chla and orthophosphate concentrations. The applicability of their data however, to north temperate lakes is questionable since: 1) water temperature in Hartbeespoort Dam did not drop below 15 °C during the winter months (unlike some north temperate lakes which may be ice-covered for >6 mo), and 2) nutrient concentrations in the reservoir are almost always well above the requirements for phytoplankton. In addition, sample sizes in their correlation analyses were small (n=16 to 20, depending on the variable) and most of the variables are autocorrelated.

To avoid the problem of autocorrelation and to use the data in a predictive manner, I developed univariate linear and a multiple regression model to predict MC-LR concentrations in phytoplankton. Of the single variables tested, TN:TP explained the most amount of variation in the MC-LR data (Table 4), although this model was based on only one year of data (1993, the only year when TN and TP were measured). I also found that

MC-LR in phytoplankton could be predicted from TP, *M. aeruginosa* biomass and Secchi depth in a multiple regression model (Table 4). The inclusion of nutrients in both the univariate and multiple regression models stresses the importance of nutrients in the prediction of MC-LR. My data supports the hypothesis that an increase in phosphorus loading (whether internally or externally mediated) or a shift in the N:P ratio may increase the incidence of toxic blooms of cyanobacteria and the concentration of toxin produced, with respect to MC-LR and *M. aeruginosa*. This is the first study to predict MC-LR concentrations in phytoplankton based on data collected from several lakes over a relatively long time frame (5 yr). This needs to be done in other regions of the world to test the generality of the models presented here.

In conclusion, there was a wide range in MC-LR concentration in the phytoplankton between the study lakes, and the concentration of MC-LR was likely regulated by both the biomass of *M. aeruginosa* in the water column and the concentration of MC-LR produced per unit mass of *M. aeruginosa*. Factors such as TP may have had an impact on both *M. aeruginosa* abundance and MC-LR produced per unit mass of the species, while factors such as turbidity and the ratio of TN:TP may have affected *M. aeruginosa* abundance only. Understanding the mechanisms of how limnological variables affect microcystin production at the cellular level is needed. This should ideally be evaluated in field studies such as the present one, mesocosm experiments (e.g., lake enclosures) and in laboratory experiments with cells isolated from the same enclosures or lakes. Perhaps our best approach to understanding how microcystin production is affected by limnological variables is to answer other related questions: why are microcystins produced/what is their function? This would go a long way in our understanding of how microcystin production is regulated in lakes.

Table 1. Water quality parameters and lake and watershed characteristics for the 12 study lakes. Water quality variables represent means for May to September, 1991 and are based on analysis of water samples collected from the surface to a depth of 2 m (or a depth of 1 m in Little Beaver Lake). TP-total phosphorus, Chla-chlorophyll *a*. Lake and watershed characteristics (maximum (Z_{max}) and mean lake depth (Z), watershed area to lake surface area ratio (W:L), water residence time (WRT)) were obtained from Mitchell and Prepas (1990). W:L and WRT for Baptiste Lake are from both basins combined and are unknown for Little Beaver Lake.

Lake/Units	Variable										
	NO ₃ ⁻ + NO ₂ ⁻ μg L ⁻¹	TP μg L ⁻¹	Chla μg L ⁻¹	SO ₄ ²⁻ μEQ L ⁻¹	Na ⁺ μEQ L ⁻¹	HCO ₃ ⁻ μEQ L ⁻¹	Conductivity μS cm ⁻¹	Z _{max} m	Z m	W:L	WRT yr
Baptiste N	16	57	24	229	1175	3065	332	15.5	5.6	29	6.0
Baptiste S	12	35	12	250	1131	2983	329	27.5	11.9		
Coal	54	103	33	937	2349	3524	498	5.5	3.5	115	0.9
Driedmeat	38	201	13	791	1697	3507	422	3.7	2.2	438	0.3
Figure Eight	29	95	32	312	131	1950	249	6.0	3.0	12	4.5
Halfmoon S	13	71	26	208	957	1639	268	8.5	4.7	6	60.0
Hastings	19	87	41	5267	4133	4556	836	7.3	2.4	31	>100
Isle	16	124	62	146	783	2459	296	7.5	4.1	11	9.5
Lac la Nonne	17	213	79	229	827	2311	314	19.8	7.8	24	18.5
Little Beaver	34	186	49	2561	5307	4458	814	2.5	2.0		
Nakamun	12	102	71	125	957	2262	275	8.0	4.5	13	21.0
Steele	20	116	27	21	305	2737	265	6.1	3.2	39	2.0
Wizard	47	63	30	104	1131	2786	310	11.0	6.2	12	13.5

Table 2. Correlations between microcystin-LR concentration (ng L^{-1}) and limnological parameters and cyanobacterial species biomass based on log-transformed data from all data combined (1990 to 1994), from 1991 data (June to October) only and combined minus 1991 data. *P* values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, n.s. not significant ($P > 0.05$). df = degrees of freedom (n-2), df for combined data - 1991 data = df combined - df 1991.

	Combined data			1991 data			Combined minus 1991	
Variable	<i>r</i>	<i>P</i>	df	<i>r</i>	<i>P</i>	df	<i>r</i>	<i>P</i>
Total Phosphorus	0.54	***	254	0.38	***	86	0.63	***
Chlorophyll <i>a</i>	0.48	***	266	0.36	***	90	0.55	***
Secchi depth	-0.38	***	262	-0.43	***	88	-0.34	***
Na ⁺	0.34	***	89	0.29	*	51	0.57	***
Color	0.33	***	88	0.35	**	52	0.45	**
pH	0.32	***	228	0.28	**	86	0.35	***
Turbidity	0.30	***	119	0.34	***	83	0.21	n.s.
Conductivity	0.21	***	180	0.29	**	86	0.17	n.s.
SO ₄ ²⁻	0.16	n.s.	53	0.09	n.s.	47	0.01	n.s.
NO ₃ ⁻ + NO ₂ ⁻	-0.11	n.s.	156	-0.18	n.s.	86	0.07	n.s.
HCO ₃ ⁻	0.07	n.s.	226	0.08	n.s.	83	0.08	n.s.
NH ₄ ⁺	0.05	n.s.	220	0.04	n.s.	84	0.08	n.s.
Water Temperature	0.00	n.s.	261	-0.16	n.s.	84	0.08	n.s.

Table 2. (continued).

	Combined data			1991 data			Combined minus 1991	
Variable	<i>r</i>	<i>P</i>	df	<i>r</i>	<i>P</i>	df	<i>r</i>	<i>P</i>
<i>M. aeruginosa</i>	0.52	***	215	0.45	***	83	0.57	***
<i>Phormidium</i> sp.	0.52	***	214	0.45	***	82	0.56	***
<i>Aph. flos-aquae</i>	0.07	n.s.	214	-0.13	n.s.	82	0.16	n.s.
<i>Gomphosphaeria</i> sp.	-0.05	n.s.	214	0.09	n.s.	82	-0.16	n.s.
<i>An. flos-aquae</i>	-0.03	n.s.	215	0.10	n.s.	82	-0.13	n.s.

Table 3. Correlations between microcystin-LR (MC-LR) concentration ($\mu\text{g g}^{-1}$ *M. aeruginosa*) and *M. aeruginosa* biomass ($\mu\text{m}^3 \text{mL}^{-1}$) and limnological parameters based on log-transformed combined data. *P* values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, n.s. not significant ($P > 0.05$). df=degrees of freedom (n-2).

Variable	MC-LR ($\mu\text{g g}^{-1}$ <i>M. aeruginosa</i>)			<i>M. aeruginosa</i> biomass		
	<i>r</i>	<i>P</i>	df	<i>r</i>	<i>P</i>	df
Secchi depth	-0.32	***	207	-0.36	***	236
Total Phosphorus	0.46	***	206	0.45	***	236
Chlorophyll <i>a</i>	0.33	***	209	0.49	***	237
Color	0.28	*	53	0.21	n.s.	60
Turbidity	0.16	n.s.	81	0.27	**	94
Na ⁺	0.26	n.s.	52	0.09	n.s.	60
Conductivity	0.27	***	138	0.02	n.s.	159
pH	0.25	***	183	0.34	***	211
NO ₃ ⁻ + NO ₂ ⁻	-0.06	n.s.	114	0.04	n.s.	134
Water Temperature	-0.12	n.s.	204	0.06	n.s.	234
SO ₄ ²⁻	0.12	n.s.	42	0.07	n.s.	47
HCO ₃ ⁻	0.08	n.s.	182	0.07	n.s.	210
NH ₄ ⁺	0.02	n.s.	182	0.03	n.s.	207
<i>M. aeruginosa</i>	0.46	***	210			

Table 4. Regression analyses of microcystin-LR (MC-LR, ng L⁻¹) with limnological parameters. Regressions are based on combined data except for regression with TN:TP (total nitrogen: total phosphorus) where regression is based on 1993 data only. *P* values: * *P*<0.05, ** *P*<0.01, *** *P*<0.005. df=degrees of freedom.

Model	Independent variable(s)	Transformation(s)	Equation of line	<i>r</i> ²	<i>P</i>	df
Linear regression	TP	log X, log Y	logMC-LR = -3.91 + 2.53log(TP)	0.29	***	1, 252
Linear regression	<i>Microcystis</i> biomass	log X, log Y	logMC-LR = -0.49 + 0.39log(<i>Microcystis</i> biomass)	0.27	***	1, 215
Linear regression	TN:TP	log X	MC-LR = 2061 - 1816log(TN:TP)	0.70	*	1, 36
Multiple regression	TP, <i>Microcystis</i> biomass, Secchi depth	log X, log Y	logMC-LR = -3.16 + 1.57log(TP) + 0.25log(<i>Microcystis</i> biomass) - 0.92log(Secchi depth)	0.66	***	3, 206

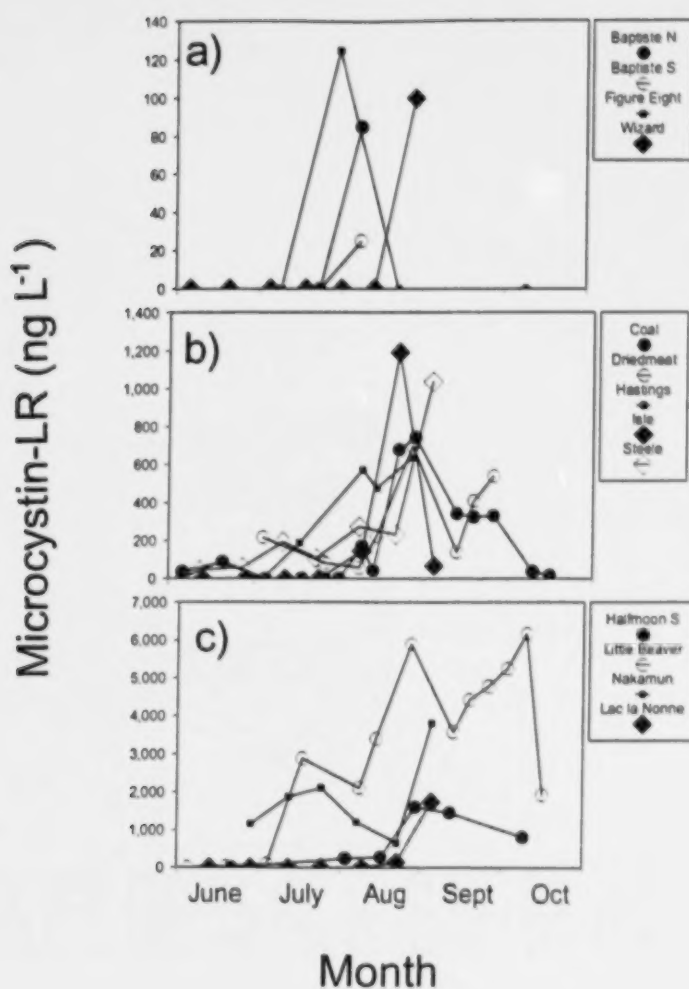


Figure 1. Microcystin-LR concentration in the phytoplankton from the 12 study lakes during 1991. Microcystin-LR concentration is expressed as ng L⁻¹ of lake water and represents cellular toxin, not dissolved in water. Phytoplankton samples for microcystin-LR analyses were collected with a plankton net from the lakes' surface (0 to 0.5 m). Lakes are divided into those with: a) low seasonal microcystin-LR concentration, b) intermediate seasonal concentration, and c) high seasonal concentration. Note the change in microcystin-LR scale between sets of lakes.

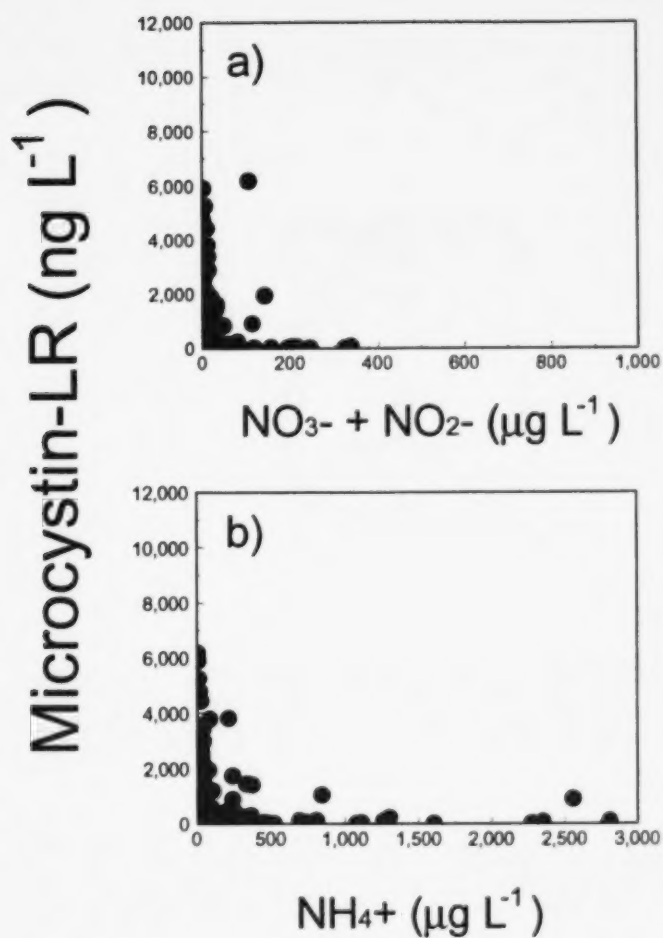


Figure 2. Relationship between microcystin-LR concentration (as ng L⁻¹) and a) NO₃⁻ + NO₂⁻, and b) NH₄⁺. Graphs are based on the combined (1990 to 1994) data.

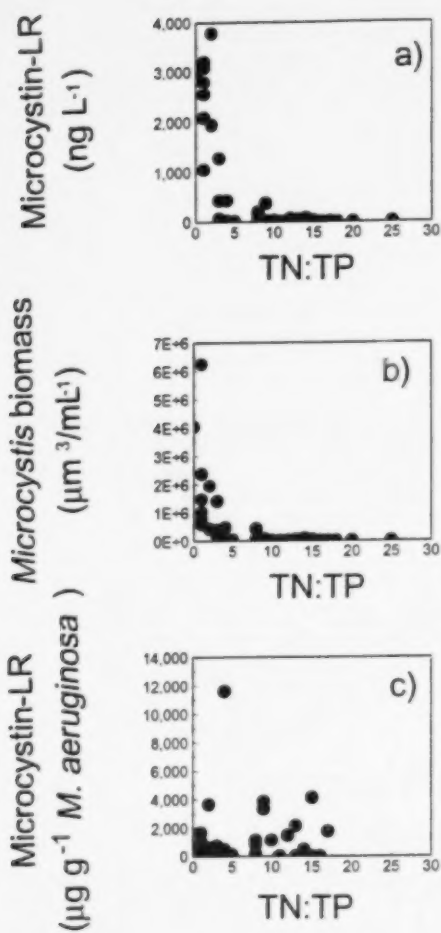


Figure 3. Relationship between a) microcystin-LR concentration (as ng L⁻¹), b) *M. aeruginosa* biomass, and c) microcystin-LR concentration (as μg g⁻¹ *M. aeruginosa*) and the ratio of total nitrogen (TN) to total phosphorus (TP). Data represent individual sampling dates during 1993 for Coal, Driedmeat and Little Beaver lakes combined.

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Chapter 6

Microcystin-LR concentration in aquatic food web compartments from lakes of varying trophic status⁴

Introduction

Microcystins are cyclic peptides produced primarily by the cyanobacterium *Microcystis aeruginosa* (Namikoshi et al. 1992; Kotak et al. 1993, 1995a). Accidental ingestion of microcystins has been responsible for the poisoning and death of livestock, wildlife and pets worldwide (Schwimmer and Schwimmer 1968; Beasley et al. 1983; Edler et al. 1985) and have also been implicated in human-related illness (Falconer 1991; Lawton and Codd 1991). Although more than 50 microcystins have been structurally characterized, microcystin-LR (MC-LR) is the most common analogue in Alberta lakes (Boland et al. 1993; Craig et al. 1993; Kotak et al. 1993). It is also one of the most toxic of the microcystins, having an LD₅₀ of 50 µg·kg⁻¹ by intraperitoneal injection in mice (Carmichael 1992).

Much of the toxicological research on the toxicity of microcystins has traditionally focussed on laboratory mammals (e.g., rats, mice). Recent studies have evaluated the relationship between toxic cyanobacteria and other components of aquatic food webs, including zooplankton and fish. Most of these studies however, were acute toxicity assays on several genera of zooplankton (Lampert 1982; Fulton and Paerl 1987; DeMott et al. 1991; Rothhaupt 1991; Gilbert 1994; Jungmann and Benndorf 1994; Reinikainen et al. 1994) and fish (Phillips et al. 1985; Sugaya et al. 1990; Rabergh et al. 1991; Kotak et al. submitted c+d). Only one study has examined whether microcystins are accumulated by natural populations of aquatic invertebrates (Watanabe et al. 1992) and none have examined *in-situ* MC-LR concentration in food web compartments simultaneously in a

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single water body or in lakes spanning a gradient in trophic status. This study reports the concentration of MC-LR in up to 5 compartments (water, phytoplankton, micro- and macroinvertebrates, and to a limited extent, fish) from up to four lakes in central Alberta, ranging in trophic status from oligo-mesotrophic to hypereutrophic. MC-LR concentrations were then related to physical and biological characteristics of the lakes.

Materials and Methods

Study lakes - The four lakes chosen for study are located in central Alberta: Coal, Driedmeat, Little Beaver and Narrow lakes. Three of the lakes (Coal, Driedmeat and Little Beaver) have been part of a study extending from 1990 to 1994 focussing on microcystin-LR (MC-LR) dynamics in phytoplankton (Kotak et al. 1993, 1995a, submitted b). For this study, Coal and Little Beaver lakes were sampled every two weeks from May to the end of September in 1992, and May to the end of August in 1993 and 1994. Driedmeat Lake was sampled similarly, although sampling in 1993 extended until the end of September. Narrow Lake was sampled only in 1994: on 13 May, 14 June, 11 July and 20 September. The lakes chosen differed in trophic status and in the potential for MC-LR production. Historically, Narrow Lake is oligo-mesotrophic, Coal Lake is eutrophic to hypereutrophic, and Driedmeat and Little Beaver lakes are hypereutrophic based on their mean summer total phosphorus and chlorophyll *a* concentrations in the euphotic zone (Mitchell and Prepas 1990; Kotak et al. 1993).

On each sampling date an integrated water sample was collected from the surface of the water to a depth of 2 m (except for Little Beaver Lake where water was collected from the surface to a depth of 1 m) at four sites with Tygon tubing fitted with a one-way valve and the water was then combined. These samples are referred to as epilimnetic. Water was analyzed for total phosphorus by colorimetric methods after potassium persulfate digestion (Menzel and Corwin 1965, as modified by Prepas and Rigler 1982), although large particles ($> 250 \mu\text{m}$) were not filtered out. Conductivity was measured on a Radiometer Analytical Model CDM83 conductance meter. Alkalinity (as $\text{mg L}^{-1} \text{CaCO}_3$) was determined on a Mettler DL21 autotitrator (APHA et al. 1992). A small subsample (10 mL) of the water was filtered through a pre-rinsed, $0.45\text{-}\mu\text{m}$ Millipore HA filter and

frozen for subsequent microcystin analysis. Phytoplankton biomass (estimated as chlorophyll *a*) was determined from water samples that were filtered onto Whatman GF/C filters, extracted in 95% ethanol and measured spectrophotometrically (Bergmann and Peters 1980). Phytoplankton were identified and enumerated on an inverted microscope (Prescott 1978) and biomass determined from comparable geometric shapes. *Microcystis aeruginosa* abundance (cells mL⁻¹) and biomass of the major phytoplankton divisions (Bacillariophyta, Chlorophyta, Cryptophyta, Cyanophyta) are reported. Secchi disk depth and water temperature were recorded at one central site at each lake. Surface (0 to 2 m or 0 to 1 m) water temperature was recorded with a YSI Model 51 temperature/O₂ meter. Total phosphorus, conductivity and Secchi disk depth for 1994 are reported as summer means from May to September $\pm$ standard error. Mean summer alkalinity ($\pm$ SE) in Coal, Driedmeat and Little Beaver lakes are reported for 1993.

In 1992 and 1993 in Coal, Driedmeat and Little Beaver lakes, and in 1994 in Narrow Lake, zooplankton were collected from the 0 to 2-m or 0 to 1-m stratum of the water column with a 243- μ m mesh plankton net. One vertical haul was made at each of four sites per lake and the four samples combined for zooplankton identification. Samples were preserved in 10% buffered formalin and zooplankton were identified and enumerated with a dissecting microscope. Identification was based on descriptions in Pennak (1978). The abundance of zooplankton (number L⁻¹) was calculated assuming the net sampled a cylinder of water with 100% efficiency. Since the sampling efficiency of plankton nets is variable and often much less than 100% (see Prepas and Vickery 1984) the abundances may be underestimates. Abundance of each species was then expressed as a percent of the total abundance in the sample. To collect zooplankton for microcystin-LR (MC-LR) analysis, a maximum of five vertical hauls were also made at four sites per lake and the four samples of concentrated zooplankton (also containing phytoplankton) were then resuspended in 1 L of lake water. Upon arrival at the laboratory, the plankton sample was gently bubbled with CO₂ to anesthetize the zooplankton. The sample was then tightly capped and the zooplankton settled to the bottom of the jar while the cyanobacteria rose to the surface. The cyanobacteria that accumulated along the surface, and most of the water were siphoned off, avoiding removal of the zooplankton on the bottom. The

remaining zooplankton and phytoplankton were re-suspended in distilled water and the process repeated a minimum of four times. By the fourth repetition the samples contained only zooplankton and groups of phytoplankton that settled out quickly (e.g., diatoms). Microscopic examination of the processed sample revealed no detectable *Microcystis aeruginosa*, the species responsible for MC-LR production in Alberta lakes (Kotak et al. 1993, 1995a, submitted b). Thus, any MC-LR detected in these samples was presumably from zooplankton tissue or gut contents. The sample was then frozen and freeze-dried. On several sampling dates zooplankton biomass was insufficient to attempt MC-LR analysis, therefore only 68 of 99 samples collected from Coal, Driedmeat and Little Beaver lakes were analyzed by HPLC.

Phytoplankton were collected for MC-LR analysis with a 64- μ m mesh plankton net at the same locations and in a similar manner as that for zooplankton. The samples collected at the four sites in each lake were combined and de-watered by passing the combined sample through a Plexiglass tube that was fitted with 64- μ m mesh on one end. The paste-like sample was frozen and freeze-dried. These samples contained both phytoplankton and zooplankton. To determine the concentration of MC-LR in the phytoplankton alone, MC-LR concentration detected in the separate zooplankton sample was subtracted from the MC-LR concentration detected in the sample containing both phytoplankton and zooplankton. The correction for the presence of zooplankton was not always possible as there were 99 phytoplankton samples but only 68 zooplankton samples, due to insufficient zooplankton biomass to attempt MC-LR analysis.

In 1994, a suite of aquatic macroinvertebrates were collected from each of the four lakes and analyzed for MC-LR. These included gastropods (*Lymnaea stagnalis*, *Physa gyrina*, *Helisoma trivolvis*), dragon fly (*Ashna* sp.), and damselfly (*Lestes* sp.) larvae, water boatmen (Family Corixidae), back swimmers (*Notonecta* sp.), predacious water beetles (*Dytiscus* sp.), scuds (*Gammarus lacustris*) and leeches (Subclass Hirudinea). Identifications were based on descriptions given in Clifford (1993). These invertebrates were collected with a dip net from the shoreline or from the littoral zone from the side of the boat. In addition, surface sediments were collected from four sites in each lake with an Eckmann Dredge and the four samples were combined. Mud was sifted through a 500-

μm sieve and chironomids (*Chironomus* spp.) were rinsed and saved for MC-LR analysis. All invertebrates (including zooplankton) were frozen, freeze-dried and then homogenized with a mortar and pestle.

On 29 and 30 August 1993, northern pike and white suckers (*Esox lucius* and *Catostomus commersoni*, respectively) were collected from Driedmeat Lake with a combination of gill and seine nets. Driedmeat Lake was chosen as the site for fish collection because I felt the fish had been exposed to high concentrations of MC-LR in the phytoplankton during the previous month. The eight northern pike collected ranged in size from 38 to 46 cm (fork length) and weighed from 445 to 814 g. The 23 suckers collected ranged in size from 32 to 42 cm and weighed from 584 to 1302 g. The fish were immediately frozen and later thawed, to remove their livers for MC-LR analysis. The fish were weighed (wet weight) and a limited examination of the gut contents of the fish was made. Livers were freeze-dried prior to MC-LR analysis.

Microcystin analyses - MC-LR in phytoplankton and aquatic invertebrates was quantified by reverse phase HPLC. Phytoplankton and zooplankton and macroinvertebrate subsamples (100 and 500 mg freeze-dried weight, respectively) were placed in 15 mL 5% acetic acid, vortexed, and ultrasonicated for 30 min. The samples were then vortexed again and left overnight. Each sample was centrifuged (30 min @ 4000g), the extract (extract A) decanted off and an additional 15 mL of 5% acetic acid was added (extract B). The sample was vortexed and allowed to sit overnight. The next morning the sample was centrifuged and the two extracts (A and B) were combined. The extract was then filtered through Whatman GF/A filters and the microcystins purified and concentrated on preconditioned (5 mL methanol, then 5 mL DDW) 1 mL Supelco LC-18 disposable cartridges. After preconditioning, the sample was slowly passed through the cartridge and interfering compounds sequentially removed by passing solvents through the cartridge (5 mL 5% acetic acid, then 5 mL DDW, then 5 mL 10% methanol). Microcystins were eluted from the cartridge in 5 mL 100% methanol. The methanol was evaporated in a Reactivap heater and the sample resuspended in 2 mL of 10mM ammonium acetate:acetonitrile (75%:25%, v:v).

Over the three years of this program, three different HPLC separation methods were utilized on two separate HPLC systems. In 1992, phytoplankton were analyzed on a HPLC consisting of Waters 6000A pumps with a Hewlett Packard HP 1040A diode array detector and HP85 data processor. Samples were run under isocratic conditions at a flow rate of 1 mL min^{-1} . The mobile phase consisted of 10 mM ammonium acetate and acetonitrile (74:26%, v:v). In 1993 and 1994 phytoplankton were analyzed on a Waters LC Module I HPLC with a model 746 data module. For both HPLCs, MC-LR detection was based on UV absorbance at 238 nm. In 1993, samples were run under isocratic conditions at a flow rate of 1 mL min^{-1} . The mobile phase consisted of 10 mM ammonium acetate: acetonitrile (76:24%, v:v). In 1994, samples were run under linear gradient conditions (20 to 30% acetonitrile in 10 mM ammonium acetate over 10 min, then at 20% from 10 min until the end of the run [i.e., 30 min]). Flow rate was 1 mL min^{-1} . A comparison of the two HPLCs and the three separation methods (two isocratic, one gradient) indicated no significant difference between the quantity of MC-LR detected, although the gradient method gave much better separation of MC-LR away from other interfering peaks (Kotak 1995). MC-LR concentration in phytoplankton was first calculated as $\mu\text{g g}^{-1}$ of phytoplankton biomass based on calibration curves of MC-LR concentration versus HPLC peak area, and the mass of sample extracted. MC-LR was then re-expressed volumetrically, as ng L^{-1} . This volumetric expression represented MC-LR in the phytoplankton cells per L of lake water, not as dissolved toxin. To calculate volumetric MC-LR concentrations, the mass of phytoplankton in the water (g L^{-1} dry weight) was estimated by use of the relationship between chlorophyll *a* and phytoplankton dry weight (conversion factor of 6.7×10^{-5} ; APHA et al. 1992) and then multiplied by $\mu\text{g g}^{-1}$ of phytoplankton biomass.

All invertebrate samples (zooplankton and macroinvertebrates) were analyzed for MC-LR on the latter HPLC system under the same gradient conditions (20 to 30% acetonitrile) as for the phytoplankton in 1994. MC-LR concentrations in the invertebrates are reported as $\mu\text{g g}^{-1}$ of biomass. In addition, the protein phosphatase (PP1c) assay was also utilized to quantify total microcystin concentration in the zooplankton samples as well as total aqueous (dissolved) microcystin concentration in water collected from the upper 2

or 1 m of the water column. This would give an indication of the quantity of MC-LR and other microcystins in the samples. The PP1c method (Luu et al. 1993) is based on the premise that microcystins inhibit the dephosphorylation of phosphorylase-a to phosphorylase-b by protein phosphatases (e.g., PP1c) in eukaryotic cells (MacIntosh et al. 1990). The assay results are expressed as total microcystin inhibition (as there may be more than 50 microcystins present in a sample) in MC-LR equivalent units. The HPLC method quantified MC-LR specifically, while the PP1c assay detected all microcystins and the data are subsequently converted to MC-LR equivalent units. The detection limit of the PP1c assay was less than 10 pg MC-LR equivalents while the detection limit for MC-LR by HPLC was 10 to 20 ng for a 20 μ L injection. For the PP1c assay, subsamples (100 to 500 mg) of zooplankton were extracted as described above for HPLC, except that the extraction solvent was 100% methanol and microcystins were not preconcentrated on LC-18 cartridges. Raw lake water samples were analyzed without prior treatment.

Livers from the fish collected from Driedmeat Lake were homogenized with a mortar and pestle and extracted in 100 mL of 100% methanol. The methanol was evaporated to approximately 10 mL and 400 mL of DDW was added. The sample was then passed through preconditioned Supelco C-18 cartridges (6 mL, 500 mg tubes) and the sample eluted in 100% methanol as described earlier. The extract was then filtered through a 0.45- μ m Millipore HV filter and injected onto a Waters LC Module I HPLC and run under gradient conditions (described above).

To confirm the presence of MC-LR (and possibly other microcystins) in the various compartments sampled, one sample of phytoplankton (collected from Driedmeat Lake on 17 August 1994), zooplankton (containing 53% *Daphnia pulex*, 20% *Acanthocyclops vernalis*, 16% *Diaphanisoma leuchtenbergianom*, 9% *Diaptomus oregonensis* and 2% *Bosmina longirostris* based on abundance, and collected from Driedmeat Lake on 11 August 1993), gastropod (*Physa gyrina* collected from Driedmeat Lake on 17 August 1994), and northern pike liver and sucker liver (collected from Driedmeat Lake on 29&30 August 1993) were fractionated by HPLC and each fraction analyzed for PP1c inhibition. Samples were run on a Waters LC Module I HPLC under the gradient conditions previously described. Between 5 and 8 (depending on the sample)

0.2 to 1.0-min fractions (i.e., 0.2 to 1.0 mL fractions) were collected over the 20 min runs. One fraction represented the retention time window where MC-LR was expected to elute from the column, and the other fractions represented other unidentified peaks and also areas in the chromatograms where no peaks occurred. The fractions were then analyzed for the ability to inhibit PP1c. Additionally, the presence of MC-LR in two invertebrate samples (the zooplankton and gastropod identified above) was confirmed by spiking the samples with a known amount of MC-LR standard.

Statistical Analysis - MC-LR concentrations in water, phytoplankton, zooplankton and gastropods are also reported as summer means (May to September) $\pm$ SE for the years in which the compartments were sampled. Correlation analyses were performed on log-transformed data with SPSS for Windows (version 6.0) statistical software.

Results

In 1994, the only year for which data were collected from all four lakes for this study, Narrow Lake was classified as oligo-mesotrophic with a mean summer epilimnetic total phosphorus (TP) concentration of $15 \pm 2 \mu\text{g L}^{-1}$. Coal, Driedmeat and Little Beaver lakes were hypereutrophic, but differed substantially from each other in mean summer epilimnetic TP concentration (Table 1): 71, 207 and $530 \mu\text{g L}^{-1}$, respectively. Narrow Lake had the most transparent water (Secchi depth averaged 7.2 m in 1994), while Coal, Driedmeat and Little Beaver lakes were much less transparent, with an average Secchi depth less than 1.5 m (Table 1). The water in the four lakes ranged from fresh (conductivity of 231 to $574 \mu\text{S cm}^{-1}$ for Narrow, Coal and Driedmeat lakes, respectively) to slightly saline ($826 \mu\text{S cm}^{-1}$ in Little Beaver Lake) in 1994 and all four lakes contained hard water (Table 1). The hypereutrophic lakes are all shallow (<6 m) and well mixed by the wind while Narrow Lake is deep (maximum depth of 39 m) and thermally stratified during summer.

From 1992 to 1994 the dominant phytoplankton taxa in the three hypereutrophic lakes were usually cyanobacteria: *Microcystis aeruginosa*, *Aphanizomenon flos-aquae*, *Gomphosphaeria* sp., *Anabaena flos-aquae* and *Phormidium* sp. (Kotak et al. 1995a, Lam 1994). The phytoplankton community in Narrow Lake during 1994 included chlorophytes

(*Clamydomonas* sp.), cryptophytes (*Rhodomonas* sp.) and chrysophytes (*Ochromonas* sp.).

On the four dates in 1994 when I sampled Narrow Lake, chlorophyll *a* was less than $2 \mu\text{g L}^{-1}$. Chlorophyll *a* in the three hypereutrophic lakes was lowest in Coal Lake, was fairly consistent between years, and peaked in August (Table 2; Fig. 1a, 2a and 3a). Chlorophyll *a* varied most between years in Little Beaver Lake (Fig. 1c, 2c and 3c). In 1992 and 1993, mean summer chlorophyll *a* in Little Beaver Lake was 33 and $30 \mu\text{g L}^{-1}$, respectively, while in 1994 it more than doubled ($67 \mu\text{g L}^{-1}$; Table 2) and on 17 August 1994 it exceeded $150 \mu\text{g L}^{-1}$. Driedmeat Lake generally had the highest chlorophyll *a* concentration of the lakes studied over the three years: mean summer concentration of $51 \mu\text{g L}^{-1}$ for all three yr (Table 2) with maxima for chlorophyll *a* over $100 \mu\text{g L}^{-1}$ in July and August of 1992 and 1993 (Fig. 1b and 2b).

Microcystin-LR (MC-LR) concentration in the phytoplankton was highest in the two most hypereutrophic lakes (Driedmeat and Little Beaver) and varied intra-annually in the two lakes. For example, within a year, MC-LR concentration in the phytoplankton from Driedmeat Lake was highest from August to early September ($1,212$ to $2,987$ in 1992 and $2,093$ to $3,780 \text{ ng L}^{-1}$ in 1993; Fig. 1e and 2e) and highest near the end of August in 1994 ($5,778$ to $6,069 \text{ ng L}^{-1}$ Fig. 3e). Peak MC-LR concentration in Driedmeat Lake phytoplankton corresponded to periods when *Microcystis aeruginosa* abundance was at its seasonal high (usually greater than $200,000 \text{ cells mL}^{-1}$ in August; Fig. 1e, 2e and 3e). Both *M. aeruginosa* abundance and MC-LR concentration in the phytoplankton also differed substantially between the three years of study in the two lakes. In Little Beaver Lake, mean summer *M. aeruginosa* abundance and MC-LR concentration in phytoplankton was $43,024 \text{ cells mL}^{-1}$ and 488 ng L^{-1} , respectively, in 1992, decreased 9 and 6 fold, respectively, in 1993, and in 1994 increased almost 15 and 57 fold relative to 1993 (Fig. 2f and 3f; Table 2). The highest MC-LR concentration measured during the three years was in phytoplankton from Little Beaver Lake ($11,216 \text{ ng L}^{-1}$) on 17 August 1994. Both *M. aeruginosa* and MC-LR were virtually absent in the phytoplankton from Coal Lake in all three years (Fig. 1d, 2d and 3d, Table 2). *Microcystis aeruginosa* was absent from Narrow Lake on three of four sampling dates; on 20 September there were a

few cells (1221 cells mL⁻¹). MC-LR was not detected in any of the phytoplankton samples from Narrow Lake (Table 2). Seasonal changes in MC-LR concentration in the phytoplankton tracked changes in *M. aeruginosa* abundance closely: the two were highly correlated ($r=0.67$, $P<0.0001$, $df=121$).

Aqueous microcystin concentration in lake water (expressed as MC-LR equivalents) was highest in Driedmeat and Little Beaver lakes in 1994. In Driedmeat Lake aqueous microcystin increased over the summer, peaking at 344 ng L⁻¹ in mid August (Fig. 4). The trend of increasing microcystin concentration in the water paralleled the trend of increasing MC-LR in the phytoplankton in Driedmeat Lake ($r=0.89$, $P=0.006$, $df=5$, Fig. 4 and 3e). In contrast, microcystin concentration in the water from Little Beaver Lake in 1994 peaked on 29 June (284 ng L⁻¹) when MC-LR concentration in the phytoplankton was low (Fig. 4 and 3f). Aqueous microcystin concentration was close to our detection limit in Narrow and Coal lakes, generally below 60 ng L⁻¹ (Fig. 4). Aqueous microcystin concentrations for all lakes combined were correlated to MC-LR in the phytoplankton ($r=0.83$, $P<0.001$, $df=24$).

MC-LR was detected by HPLC in 27 of the 68 zooplankton samples collected; these 27 samples came from Driedmeat and Little Beaver lakes, the lakes containing the highest MC-LR concentrations in phytoplankton and water. MC-LR concentrations in the zooplankton communities peaked in August in the former (Fig. 1h and 2h), but was highest from mid-May to late August in the latter lake (Fig. 1i and 2i). The peaks in MC-LR concentration in the zooplankton communities coincided with peak MC-LR in phytoplankton ($r=0.69$, $P<0.001$, $df=66$), although there were occasions where peak MC-LR in zooplankton did not correspond to maximum MC-LR concentration in the phytoplankton (e.g. MC-LR concentration in the zooplankton in Driedmeat Lake was equally high on 25 May 1993 [15 µg g⁻¹] as those samples collected in August 1993 [up to 17 µg g⁻¹] and yet MC-LR concentration in the phytoplankton on 25 May was up to 209-times lower than in August [Fig. 2e+h]).

In Little Beaver Lake, substantive shifts in zooplankton community composition occurred and MC-LR concentration decreased in the zooplankton after periods when MC-LR concentration was at its highest in the zooplankton community. In 1992, up to a 17-

fold decrease in MC-LR concentration in the zooplankton community (Fig. 1j), coincided with a shift from *Daphnia pulex* (in June and July) to *Diaptomus oregonensis* and *Diaphanisoma leuchtenbergianom* in July and August (Fig. 1l). In contrast, similar shifts in zooplankton community structure were not observed in Driedmeat Lake in 1992 and 1993 (Fig. 1k+2k, respectively). In addition, no single zooplankton species was dominant during periods when MC-LR concentration in the zooplankton community peaked.

For the majority of zooplankton samples (i.e., 61 of 68 samples) there was agreement between total microcystin and MC-LR concentrations determined by PP1c assay and HPLC, respectively (Fig. 5). These results are consistent with observations that the major microcystin produced by phytoplankton in Alberta lakes is MC-LR (Kotak et al. 1993; Boland et al. 1993; Craig et al. 1993). In five of six samples where total microcystin concentration was higher than MC-LR detected by HPLC, the chromatograms contained other peaks before and after the peak corresponding to MC-LR. In the case of two samples, for which total microcystin concentration by the PP1c assay was 3-fold higher than MC-LR by HPLC, the additional peaks were equivalent to or larger than the MC-LR peak. Lastly, one zooplankton sample, collected from Driedmeat Lake on 9 June 1993, which did not contain detectable MC-LR by HPLC, had a total microcystin concentration of $52 \mu\text{g g}^{-1}$ (Fig. 5). The HPLC chromatogram for this sample had a very noisy baseline. There was no peak corresponding to the MC-LR retention time, but there was another peak eluting 2 min after the known retention time of MC-LR. Therefore, occasionally other microcystins were present in the plankton which were not quantified by HPLC.

MC-LR was detected in three species of gastropods (*Lymnaea stagnalis*, *Helisoma trivolvis* and *Physa gyrina*) collected from Driedmeat and Little Beaver lakes only in August (Fig. 3h+i) when MC-LR in the phytoplankton was at its highest (Fig. 3e+f). MC-LR was not detected in the gastropods collected earlier in the year in these two lakes, nor from those collected from Coal and Narrow lakes, or in any other macroinvertebrates. On 3 and 17 August in Driedmeat Lake, *P. gyrina* had the highest MC-LR concentration (90 and $121 \mu\text{g g}^{-1}$, respectively) while on 23 August *L. stagnalis* had the highest concentration ($96 \mu\text{g g}^{-1}$). In Little Beaver Lake, MC-LR was detected

only once in *L. stagnalis* (on 17 August; $54 \mu\text{g g}^{-1}$) and *H. trivolvis* (on 23 August; $11 \mu\text{g g}^{-1}$). No samples of *H. trivolvis* and *P. gyrina* on 17 August and *L. stagnalis* and *P. gyrina* on 23 August were collected. The non-detectable values, indicated by an asterisk in Fig. 3h+i, represent non-detectable MC-LR for all three gastropod species. Detectable MC-LR concentrations in the gastropods from Driedmeat Lake also coincided with high aqueous total microcystin in the lake water (Fig. 4). However, the same was not true in Little Beaver Lake, where MC-LR was non-detectable in gastropods even when total dissolved microcystin concentration was highest (from late June onward). Therefore, MC-LR detected in gastropods likely originated from the phytoplankton, not from toxin taken up from an aqueous source.

Northern pike and white suckers were collected from Driedmeat Lake after MC-LR had been at its highest in the phytoplankton for almost one month (average: 2849 ng L^{-1} from 4 to 30 August; Fig. 2e). Eight livers from the northern pike and 23 from white suckers were extracted and analyzed for MC-LR by HPLC. MC-LR was not detected in any of the livers from the two species of fish, despite stomach contents which suggested that the fish should be exposed to the toxin through an oral route. For example, the stomachs of the northern pike contained gastropods (which were collected from Driedmeat Lake on the same days as the fish and contained $58 \mu\text{g g}^{-1}$ total microcystin by PP1c assay) as did the stomachs of the white suckers, in addition to unidentified phytoplankton.

HPLC-linked PP1c bioassay was used to confirm the presence of MC-LR and possibly other microcystins in several samples. The HPLC chromatogram for the Driedmeat Lake phytoplankton sample contained one major peak with several smaller peaks (Fig. 6a). The largest peak in the chromatogram, with a retention time of 8.60 min, corresponded to the retention time of the MC-LR standard (Fig. 6b). Eight fractions were collected over the 20 min run, four of which inhibited protein phosphatase (Fig. 6a). The fraction with the greatest inhibition corresponded to the MC-LR peak. In addition, another peak, with a retention time of 9.07 min also inhibited PP1c. Two other peaks, both preceding MC-LR, had much lower PP1c inhibition (Fig. 6a). Therefore, in this

sample, the major microcystin in the phytoplankton was MC-LR, although another peak (likely another microcystin) also inhibited PP1c.

Several peaks were also evident in the HPLC chromatogram for the zooplankton sample analyzed by HPLC-linked PP1c bioassay (Fig. 7a). A peak with a retention time of 8.57 min corresponded with the retention time of the MC-LR standard. In addition, there was another distinct, but smaller peak at 10.00 min. Six fractions were collected and subjected to PP1c analysis. There were two fractions that inhibited PP1c, corresponding to the two above mentioned peaks in the chromatogram (Fig. 7a). A large, broad peak at 11.99 min did not inhibit PP1c. The sample was then spiked with $3.54 \mu\text{g mL}^{-1}$ MC-LR and re-run on the HPLC. The peak area of the presumed MC-LR peak in the sample increased by 1.78 fold (Fig. 7b), a recovery of 98.7% of the added MC-LR standard. This suggested that the original peak (at 8.57 min) in the sample was MC-LR. The gastropod sample contained one large, distinctive peak with a retention time of 8.56 min (Fig. 7c). Two smaller peaks (retention times of 10.11 and 12.43 min) were also present. Only one of these (at 8.56 min), with a retention time virtually identical to the MC-LR standard, inhibited PP1c (Fig. 7c). The gastropod sample was then spiked with $3.72 \mu\text{g mL}^{-1}$ MC-LR and the peak area of the presumed MC-LR peak in the sample increased 1.59 fold, a recovery of 99.6% of the added MC-LR standard (Fig. 7d). Therefore, the main peak in the chromatograms for the phytoplankton, zooplankton and gastropod samples, identified as MC-LR by HPLC retention time, also inhibited PP1c the most.

Livers from two fish (white sucker and northern pike), with non-detectable MC-LR by HPLC, were analyzed by HPLC-linked PP1c assay. A small peak with a retention time of 10.85 min was present in the sucker liver chromatogram (Fig. 8a), but this peak (or any other of the 5 fractions collected) did not inhibit PP1c. This included a fraction which covered the MC-LR retention time window. Several peaks were also present in the chromatogram from the northern pike liver (Fig. 8b) although there was no peak with a retention time corresponding to the MC-LR standard. Two fractions did inhibit PP1c marginally, one which was collected from a time window (5 to 6 min) where there was no distinguishable peak. The other PP1c-inhibiting fraction was collected around the peak with a retention time of 10.76 min, not corresponding to any of the peaks showing

inhibition from the Driedmeat Lake phytoplankton sample (i.e., not corresponding to retention times of the microcystins identified in the phytoplankton sample). The fraction that corresponded to the MC-LR retention time window did not inhibit PP1c. Therefore, MC-LR was not detected by either HPLC or HPLC-linked PP1c assay in the livers of northern pike and white sucker.

Discussion

Few studies have examined whether cyanobacterial toxins can accumulate in aquatic food webs, at what concentrations they are present in the various compartments, and how trophic status of a water body may influence toxin concentrations. In companion studies, I have shown that microcystin-LR (MC-LR) concentration in phytoplankton can be highly dynamic, reflecting, at least partially, changes in the abundance or biomass of *M. aeruginosa* within a lake, temporally within and between years, and even over 24 h (Kotak et al. 1993, 1995a, submitted b). Therefore, the occurrence of MC-LR in food web compartments will depend mainly on the occurrence and biomass of *M. aeruginosa* in the phytoplankton. Data from the present study indicates that lakes of low productivity (such as oligo-mesotrophic Narrow Lake) and even some hypereutrophic lakes (such as Coal Lake) may have a low potential for supporting microcystin-producing phytoplankton. Kotak et al. (submitted b) have shown that consistently low MC-LR concentrations in Coal Lake phytoplankton may be related to seasonal total nitrogen to total phosphorus (TN:TP) ratios that exceed 5:1, whereas higher MC-LR concentrations in Driedmeat and Little Beaver lakes correspond to TN:TP ratios that are often less than 5:1. Trophic status, therefore, likely will influence the occurrence and concentration of MC-LR in aquatic food webs by restricting the distribution of *M. aeruginosa*.

Aqueous microcystin concentration and MC-LR concentration in the phytoplankton were both highest in Driedmeat and Little Beaver lakes. Microcystins are considered endotoxins since the majority of the toxin is found within the cells. When the cell wall of the cyanobacterium is compromised, as a result of chemical treatment (Kenefick et al 1993; Lam and Prepas 1995; Lam et al. 1995a+c) or natural senescence (Berg et al. 1987), a pulse of microcystins is released into the water. Despite the much

higher MC-LR concentrations in the phytoplankton in Little Beaver than in Driedmeat Lake, concentrations of aqueous microcystin in the two lakes were similar. Dilution and/or degradation by microbial action (Lam et al. 1995b) or photolysis (Tsuji et al. 1994) may account for the similar microcystin concentrations in the two lakes.

Aquatic invertebrates analyzed for MC-LR by HPLC covered a diversity of trophic feeding levels, from detritivores (such as *Gammarus lucustris* and *Chironomis* spp.), herbivores (zooplankton such as *Daphnia pulex*, *Bosmina longirostris* and *Diaphanisoma leuchtenbergianom* and gastropods such as *Physa gyrina*, *Lymnaea stagnalis* and *Helisoma trivolvis*) to predators (zooplankton such as *Acanthocyclops vernalis* and the predacious water beetle, *Dytiscus* sp.). In general, the highest MC-LR concentrations in the zooplankton corresponded fairly well to high concentrations in the phytoplankton although there were exceptions. The correlation between MC-LR concentration in the zooplankton and phytoplankton ($r=0.69$) suggests that MC-LR may be present in the zooplankton as result of filter feeding on the toxin-containing *M. aeruginosa* cells. If so, there should be a relationship between the abundance of herbivorous grazers and MC-LR in the zooplankton. MC-LR concentration in the zooplankton was correlated to the relative abundance of all the grazers combined (*D. pulex*, *B. longirostris*, *D. leuchtenbergianom*, and *D. oregonensis*, $r=0.35$, $P<0.05$, $df=36$) but not to individual species ($P>0.09$). I also cannot eliminate the possibility that *A. vernalis* (a predatory copepod), may have been partially responsible for the detected MC-LR during these periods as MC-LR accumulation could have resulted from feeding on other genera which contained the toxin, or obtaining toxin directly from the water. However, I found no relationship between MC-LR in the zooplankton and the relative abundance of *A. vernalis* ($P=0.18$). If the toxin was obtained by filter feeding activity then a relationship between MC-LR concentration in the zooplankton and factors which have been shown to affect filtering rates in zooplankton may be noted: water temperature and algal biomass (chlorophyll *a*) in the water. However, MC-LR concentration in the zooplankton was not correlated with either water temperature or chlorophyll *a* ($P=0.13$ and 0.87 , respectively).

In addition to obtaining MC-LR by filter feeding on the toxin-containing *M. aeruginosa* cells, MC-LR may have been taken up in the aqueous form, directly from the

water. Unfortunately, no water samples were collected in 1992 and 1993 for aqueous microcystin when the zooplankton were collected. However, the absence of detectable MC-LR in macroinvertebrates (damselfly and dragon fly larvae, chironomids for example) collected in 1994 from the lakes with the highest aqueous microcystin concentrations (Driedmeat and Little Beaver), suggests that either MC-LR is not taken up from the water, or that given its high water solubility, the toxin is not retained in fatty tissues, but is rapidly eliminated. DeMott et al. (1991) found that purified, aqueous MC-LR was lethal to *Daphnia pulicaria*, *D. hyalina*, *D. pulex* and *Diaptomus birgei*, although concentrations of MC-LR used in their laboratory trials were very high (500 to 50,000 $\mu\text{g L}^{-1}$) compared to the total aqueous microcystin concentrations reported in lakes from the present study (maximum concentration of 0.34 $\mu\text{g L}^{-1}$). Therefore MC-LR must be taken up by zooplankton, presumably across respiratory surfaces from the water. There have been no studies that have examined the clearance rates of purified microcystins in zooplankton. Given that aqueous microcystin can be taken up by zooplankton, but that the high water solubility of the toxin likely precludes any "bioaccumulation" in tissues, I believe that the MC-LR detected in the zooplankton samples from the lakes in the present study was toxin that was present in the ingested *M. aeruginosa* cells, not toxin accumulated in zooplankton tissue.

Only one other study has quantified cyanobacterial toxin concentrations in zooplankton communities *in situ*. Watanabe et al. (1992) detected concentrations of microcystins -LR, -YR and -RR from 75 to 1387 $\mu\text{g g}^{-1}$ of the three analogues in zooplankton samples collected from Lake Kasumigaura, Japan. These concentrations are up to nine times higher than the highest total microcystin concentration detected by PP1c and up to 20 times higher than the highest MC-LR concentration detected by HPLC from zooplankton collected during the present study. Watanabe et al. (1992) suggested that *Bosmina fatalis* was the principle zooplankton species responsible for accumulation of the three microcystin variants, although they did not attempt to quantify the relationship between microcystin concentrations and zooplankton species composition.

It is widely accepted that, due to their poor nutritional quality, difficulties in manageability and possibly their toxicity, cyanobacteria are not utilized heavily as food by

filter feeding zooplankton (Arnold 1971; Lampert 1982; Paerl 1988). However, given the detectable concentrations of MC-LR in the zooplankton from Driedmeat and Little Beaver lakes, it appears that zooplankton feed on toxic *M. aeruginosa*. In addition to MC-LR concentration in the phytoplankton and changes in species composition of the zooplankton, the variable MC-LR concentrations in the zooplankton from Driedmeat and Little Beaver lakes might also be explained by a combination of selective feeding of the zooplankton in the lakes, and the variable abundance of alternative food sources available throughout the summer. For example, Reinikainen et al. (1994) found that *Daphnia* survival increased dramatically with increasing concentration of an alternative food source (*Scenedesmus obtusiusculus*) even when the concentration of toxic *M. aeruginosa* was high (70,000 cells mL⁻¹). One could extrapolate from this study that when alternative food sources are limited in a lake, a greater proportion of toxic *M. aeruginosa* cells, and hence, MC-LR would be ingested. To investigate this, I examined the correlations between MC-LR concentration in the zooplankton (for dates when MC-LR was detected in phytoplankton) and the biomass of different phytoplankton groups (Cyanophyta, excluding *M. aeruginosa*, Chlorophyta, Cryptophyta and Bacillariophyta). If alternative food sources were important in determining the proportion of *M. aeruginosa* grazed upon, and hence MC-LR concentration in the zooplankton, I expected to see a negative relationship between MC-LR in zooplankton and the biomass of alternative food sources. MC-LR was not correlated to the biomass of diatoms, other cyanobacteria, chlorophytes and cryptophytes ($P=0.08, 0.17, 0.21$ and 0.43 , respectively).

The concentration of natural toxins such as microcystins in zooplankton communities in lakes is likely a result of a complex interaction among several factors. Foremost, this might include the variable concentration of toxin in the phytoplankton. In addition, species composition of the zooplankton community would be expected to influence MC-LR concentration in the community by affecting species-specific filtering rates, physiological resistance to the toxins, food item preferences, and depuration rates of the toxins. Water temperature would also be expected to affect MC-LR concentration, given its importance in regulating filter feeding and digestion, and possibly depuration rate of the toxins.

It is not known whether the concentrations of MC-LR detected in the zooplankton from the two lakes were sufficient to cause mortality in the various zooplankton species, and hence cause changes in zooplankton community composition. Given the results of DeMott et al. (1991), it is unlikely that the aqueous microcystin concentrations in Driedmeat and Little Beaver lakes would cause mortality in any of the zooplankton species found. In addition, high MC-LR concentrations in the zooplankton in Driedmeat Lake during late August in 1992 (Fig. 1h) and during late May and throughout August in 1993 (Fig. 2h) were not preceded by major shifts in zooplankton species composition (Fig. 1k and 2k, respectively). It therefore did not appear that the high MC-LR burdens resulted in a change in zooplankton species composition in Driedmeat Lake. However, large shifts in zooplankton species composition did occur in Little Beaver Lake in 1992 and 1993 following peak MC-LR concentrations in the zooplankton (Fig. 1i and 1, and 2i and 1). MC-LR or other factors may have caused a shift in the species composition of zooplankton from Little Beaver Lake. It has been suggested that cyanobacterial toxins are produced as a defense mechanism against grazing pressure exerted by herbivorous zooplankton (Lampert 1981; DeMott and Moxter 1991) and may therefore control zooplankton competitive relations and species composition (Fulton and Paerl 1988). In response, the zooplankton have in turn evolved mechanisms to reduce their susceptibility to the toxins. These mechanisms include increased physiological resistance to the toxins themselves (DeMott et al. 1991), reduction in filter feeding activity (Lampert 1981, 1982) and rejection of toxic forms over non-toxic forms after the cells have been captured (DeMott and Moxter 1991). Any one of these mechanisms could also help explain the variable concentrations of MC-LR detected in the zooplankton from Driedmeat and Little Beaver lakes.

MC-LR was also detected in three species of gastropods collected from Driedmeat and Little Beaver lakes in 1994 (Fig. 3h-i). In Driedmeat Lake, MC-LR was not detected in the gastropods until early August. MC-LR concentration in *Lymnaea stagnalis* increased over the three sampling dates in August, while MC-LR concentrations in *Physa gyrina* peaked on the second sampling date (17 August; Fig. 3h). The changes in MC-LR concentration in both species follow the changes in MC-LR concentration in the

phytoplankton fairly well. In contrast, MC-LR concentration in *Helisoma trivolvis* was highest on the first sampling date in August and declined thereafter (Fig. 3h). On the last sampling date, 23 August, many of the *H. trivolvis* were dead or appeared unhealthy. It is unknown whether the high MC-LR concentrations in the phytoplankton were responsible for the large number of dead *H. trivolvis* on this date. However, since both *L. stagnalis* and *P. gyrina* appeared healthy, *H. trivolvis* may have been more susceptible to MC-LR than the other two species. Gastropods in general are not considered filter feeders (Calow 1970; Elder and Collins 1991), preferring to graze on periphyton (attached algae) which are adherent on the surfaces of macrophytes. The source of the MC-LR in the gastropods in the present study is likely from *M. aeruginosa* cells and colonies which settled out of the water column and became entangled in the abundant macrophytes in Driedmeat and Little Beaver lakes. The gastropods were almost always associated with dense growths of *Potamogeton pectinatis*, *P. richardsonii* and *Myriophyllum spicatum* which had dense films of *M. aeruginosa* on their surfaces.

Only one study has examined microcystin accumulation in macroinvertebrates. Eriksson et al. (1989) examined the accumulation of a microcystin analogue (detected by HPLC) by the freshwater mussel, *Anodonta cygnea*, exposed to a hepatotoxic laboratory culture of the cyanobacterium *Oscillatoria agardhii*. Although the mussels accumulated high concentrations of the microcystin in the tissue ($70 \mu\text{g g}^{-1}$ after a 15 day exposure), they were also exposed to high concentrations of the microcystin in pure culture (2000 to $3000 \mu\text{g toxin g}^{-1}$ or 40 to $60 \mu\text{g toxin L}^{-1}$). Gastropods from the present field study were exposed to much lower concentrations of MC-LR. The highest concentration of MC-LR in the phytoplankton during our study was $11.2 \mu\text{g L}^{-1}$. Therefore, it may be difficult to extrapolate the results of Eriksson et al. (1989) to natural lake settings. Eriksson et al. (1989) also examined the concentration of the toxin in different organs of the mussels. They found the highest concentration of the microcystin in the hepatopancreas ($>120 \mu\text{g g}^{-1}$), followed by the intestine and gonads. Little microcystin was detected in the muscle. Concentrations of MC-LR reported in our study represent whole gastropod tissue concentrations (minus the shell).

Accumulation of another cyanobacterial hepatotoxin, nodularin, has been studied in blue mussels (*Mytilus edulis*) during a hepatotoxic bloom the cyanobacterium *Nodularia spumigena* in Peel-Harvey estuary, Western Australia (Falconer et al. 1992). Gut contents of the mussels contained filaments of *Nodularia spumigena* and the highest toxicity (detected by mouse bioassay) was associated with the gastrointestinal tract of the mussels. No toxicity was observed for non-intestinal tissues, although detection of cyanobacterial hepatotoxins by the mouse bioassay method is far less sensitive than other types of detection methods, such as HPLC (see Kotak et al. 1995a).

MC-LR was not detected in the livers of fish collected from Driedmeat Lake, despite the fish potentially being exposed for more than one month in 1993 to high concentrations of MC-LR in the phytoplankton, aqueous microcystin, and MC-LR in food items (e.g., gastropods). Most of the microcystins in the fish livers may have been covalently bound to protein phosphatases, and therefore would not be detected by either HPLC or PP1c assay. The absence of detectable microcystin in the liver (or in the flesh or any other organ, data not shown) does not mean that the fish were not exposed to the toxins, or that the toxin had no health effect on the fish. Binding of any microcystin to protein phosphatases in the liver is a pathological result in itself. This would likely be manifested as focal, multifocal to massive zones of coagulative or liquifactive hepatocyte necrosis, as is seen in rainbow trout that have been exposed to MC-LR by intraperitoneal injection (Phillips et al. 1985; Kotak et al. submitted c) or oral exposure (Kotak et al. submitted d) in laboratory experiments. Similar liver lesions have also been reported in laboratory trials with carp (Rabergh et al. 1991) and goldfish (Sugaya et al. 1990). The livers of the fish collected from Driedmeat Lake were not examined for lesions by light or electron microscopy. Although long-term (i.e., > 96 h) exposure of fish to microcystins has not been studied, I would hypothesize that chronic exposure of natural fish populations to microcystins may lead to severe necrosis of hepatocytes, loss of liver function, and perhaps liver tumour promotion or mortality. Such physiological stress would clearly have an impact on growth rates in the fish. Interestingly, northern pike from Driedmeat Lake are among the slowest-growing populations in productive Alberta lakes (Mitchell and Prepas 1990). It has been speculated that the slow growth rates are due to hypoxic

stress from wide diurnal swings in dissolved oxygen and hypoxic conditions during the winter, although microcystins may also play a role. Beveridge et al. (1993) found that tilapia and silver carp, which feed directly on phytoplankton, had significantly lower opercular beat rates and hence, a lower volume of water and food particles that were passed over the gills when exposed to microcystin-containing *M. aeruginosa* cells versus non-toxic cells. Therefore, certain species may be able to avoid ingesting toxic cells through modification in feeding behavior.

There has been one study published that has confirmed that microcystins can have a substantive, and at times, lethal impact on commercially important fish populations. Kent et al. (1988) and Kent (1990) reported severe liver lesions, which they termed netpen liver disease (NLD), and mortality in netpen-reared Atlantic salmon since 1986. The liver lesions (Kent et al. 1988; Kent 1990) were identical to those caused by intraperitoneal exposure of the salmon to MC-LR in laboratory experiments (Andersen et al. 1993). The disease in fish held in netpens was not related to diet, could not be experimentally transmitted (therefore was not due to viral or bacterial action), was not related to anthropogenic contamination and became progressively worse over the summer in each year. Subsequently, Andersen et al. (1993) successfully isolated a compound from the livers of salmon inflicted with NLD and from biota fouling the netpens (utilizing HPLC-linked PP1c bioassay) that was chromatographically-indistinguishable from MC-LR. This compound was not detected in the livers of fish not afflicted with NLD. The more sophisticated extraction and cleanup steps used in the isolation and purification of MC-LR from the the salmon livers (including semi-preparative HPLC followed by the use of a microbore analytical column) compared to my method of sample extraction and cleanup, and the much greater mass of liver tissue extracted (22.7 g dry weight versus 2-3 g in the present study) may explain why I did not detect MC-LR in the livers of northern pike and white sucker. Andersen et al. (1993) is the first study to document a direct (and severe) effect of MC-LR on fish populations in the field. They suggested that the fish were exposed to the MC-LR via feeding on as yet unidentified organisms fouling the netpens.

Given the presence of MC-LR in many of the compartments of the aquatic food webs in two of the hypereutrophic lakes in our study, and the results of Andersen et al.

(1993) there is a potential for transfer and pathological effects of microcystins in aquatic food webs. However, this transfer is not likely to occur in a similar fashion as that of much more lipophilic compounds such as dioxins and furans. Bioaccumulation of the above anthropogenic contaminants occurs primarily via accumulation of the contaminants in fatty tissues. Since microcystins are considered more water soluble than dioxins and furans, I would expect little "bioaccumulation" to occur in tissues, with the exception of accumulation in the liver, in organisms occupying higher trophic levels in food webs. For example, MC-LR present within *M. aeruginosa* cells in the gut of zooplankton, could be transferred to small forage fish that feed directly on the zooplankton. Of the MC-LR that is not excreted by the forage fish, most will probably end up being localized in the liver. The majority of this microcystin would then be bound covalently to protein phosphatases, eliciting some degree of pathological effect, depending on the dose. A larger piscivorous fish preying on the smaller forage fish would not likely be affected by the MC-LR present in the liver of the forage fish since the toxin would be bound to protein phosphatases. Likewise, one could extrapolate the situation to humans or terrestrial animals which might eat the piscivorous fish. I therefore believe that transfer of microcystins to higher trophic levels in aquatic food webs may occur over a limited number of trophic levels, possibly terminating with the first organism that contains a liver or similar organ (e.g., hepatopancreas in some invertebrates). Therefore, there is also a potential for pathological effects of the toxin in a number of organisms on different trophic levels in aquatic food webs.

In addition, there is a strong possibility of transfer of MC-LR from aquatic food webs to terrestrial food webs, by waterfowl and shorebirds, for example, feeding on contaminated gastropods and zooplankton. This has not received any attention. Terrestrial animals and birds can also be exposed to the microcystins by drinking water and cells from ponds and lakes that contain toxin-producing cyanobacteria. Given this scenario, more attention needs to be directed to the ecology of toxin-producing cyanobacteria, and the role of the toxins in food webs. In particular, information on the uptake, organ-specific accumulation, and depuration of microcystins in invertebrates (zooplankton, gastropods) and fish (natural populations of northern pike, etc. and

standard laboratory toxicity assay species such as rainbow trout and fathead minnows) is needed, as well as detailed work on the pathological effects (histology and cellular ultrastructure) of the toxins.

Table 1. Water quality and lake morphometric characteristics of the study lakes. Water quality parameters (total phosphorus, Secchi disk depth, conductivity and alkalinity) represent seasonal averages (May to September) for 1994 and are based on samples collected from the surface of the water to a depth of 2 m (surface to 1 m for Little Beaver Lake), except where indicated. Values in parentheses are the standard error of the mean. Lake morphometry data were obtained from Mitchell and Prepas (1990) and Kotak et al (1993).

Variable	Units	Lake			
		Narrow	Coal	Little Beaver	Driedmeat
Total Phosphorus	$\mu\text{g L}^{-1}$	15 (2)	71 (17)	207 (26)	530 (119)
Secchi depth	m	7.2 (0.5)	1.3 (0.1)	0.8 (0.2)	1.1 (0.3)
Conductivity	$\mu\text{S cm}^{-1}$	231 (41)	474 (12)	826 (65)	574 (14)
Alkalinity ^{*1}	$\text{mg L}^{-1} \text{CaCO}_3$	155 (1)	196 (6)	332 (11)	210 (10)
Surface area	km^2	1.1	11	0.75	17
Max. depth	m	39	5.5	2.0	3.7

^{*1} Alkalinity in Narrow Lake is for 1983 (Mitchell and Prepas 1990) and for 1993 in Coal, Little Beaver and Driedmeat lakes.

Table 2. Chlorophyll *a*, *Microcystis aeruginosa* abundance and microcystin-LR concentration in various food web compartments from 1992 to 1994 in up to four study lakes. Values represent seasonal means from May to September ($\pm SE$).

Variable	Narrow				Coal				Little Beaver				Driedmeat			
	1994	1992	1993	1994	1992	1993	1994	1994	1992	1993	1994	1994	1992	1993	1994	1994
Chlorophyll <i>a</i> ($\mu\text{g L}^{-1}$)	1.1 (0.3)	1.5 (2)	10 (1)	23 (4)	33 (5)	30 (5)	67 (24)		42 (6)	61 (7)	50 (8)					
<i>M. aeruginosa</i> ($\times 10^3$ cells mL^{-1})	0.3 (0.3)	1.5 (0.3)	0.1 (0.06)	0.2 (0.1)	43.0 (8.8)	4.9 (2.5)	71.8 (37.1)		38.9 (10.4)	96.5 (32.5)	63.6 (19.3)					
MC-LR in phytoplankton (ng L^{-1})	0 (0)	0 (0)	9 (3)	45 (29)	488 (123)	78 (32)	4426 (1790)		807 (226)	1811 (334)	2935 (888)					
Aqueous MC (ng L^{-1})	42 (6)	N.S.	N.S.	67 (13)	N.S.	N.S.	204 (26)		N.S.	N.S.	150 (40)					
MC-LR in zooplankton ($\mu\text{g g}^{-1}$)	0 (0)	0 (0)	0 (0)	N.S.	11 (3)	3 (0.9)	N.S.		12 (6)	8 (2)	N.S.					
MC-LR in gastropods ($\mu\text{g g}^{-1}$) ^{*1}	0 (0)	N.S.	N.S.	0 (0)	N.S.	N.S.	11 (9)		N.S.	N.S.	23 (8)					

^{*1} average for all three gastropod species (*Lymnaea stagnalis*, *Helisoma trivolvis*, *Physa gyrina*) combined.

N.S. = not sampled.

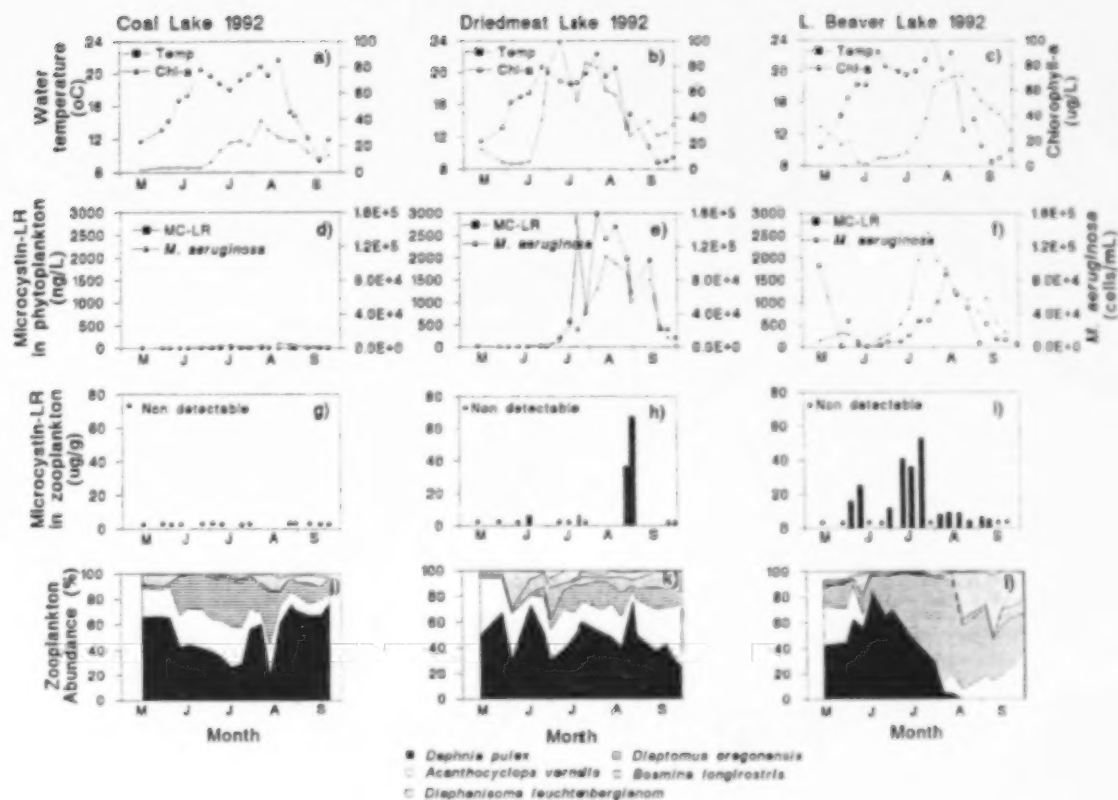


Figure 1. Seasonal changes in a-c) surface water temperature and chlorophyll *a*, d-f) *Microcystis aeruginosa* abundance and microcystin-LR concentration in the phytoplankton, g-i) microcystin-LR concentration in the zooplankton community, and j-l) zooplankton species composition for Coal (left panel), Driedmeat (middle panel), and Little Beaver (right panel) lakes for 1992. Samples were collected from the 0 to 2-m stratum of the water column in Coal and Driedmeat lakes, and from the 0 to 1-m stratum in Little Beaver Lake. Microcystin-LR was quantified by HPLC. Asterisks (*) in g-i) indicate non-detectable microcystin-LR ($<1 \mu\text{g g}^{-1}$ microcystin-LR).

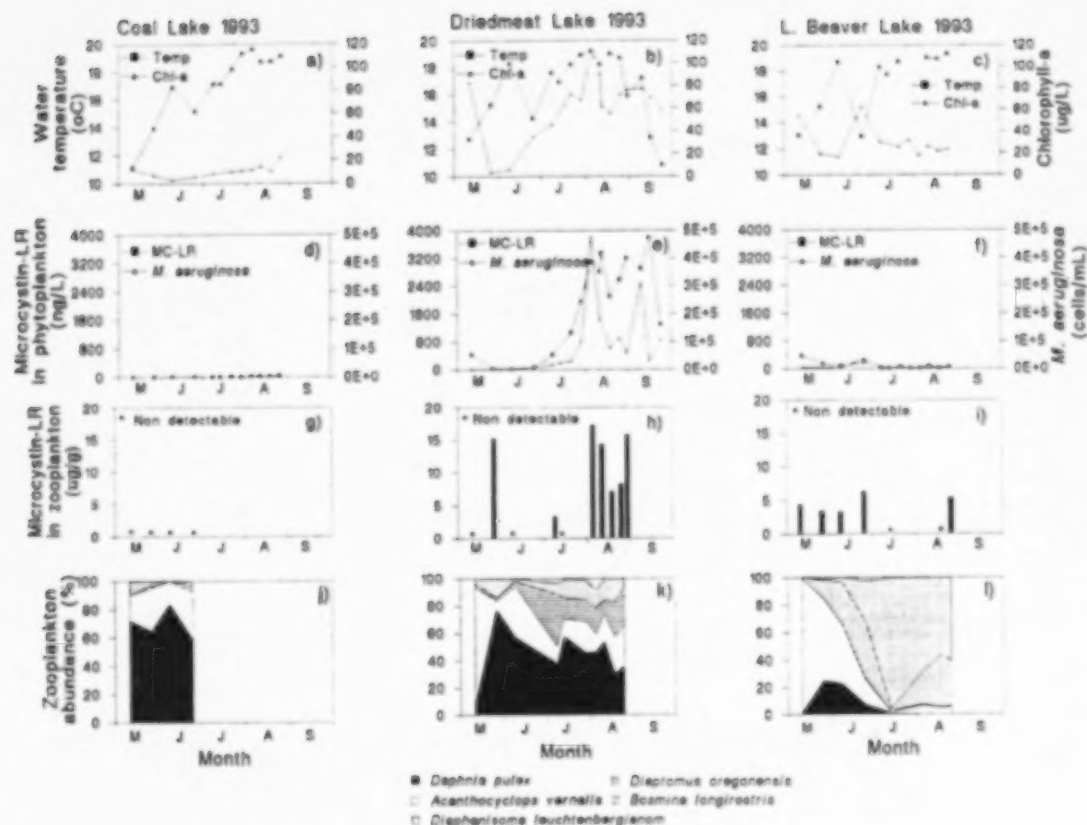


Figure 2. Seasonal changes in a-c) surface water temperature and chlorophyll *a*, d-f) *Microcystis aeruginosa* abundance and microcystin-LR concentration in the phytoplankton, g-i) microcystin-LR concentration in the zooplankton community, and j-l) zooplankton species composition for Coal (left panel), Driedmeat (middle panel), and Little Beaver (right panel) lakes for 1993. Samples were collected from the 0 to 2-m stratum of the water column in Coal and Driedmeat lakes, and from the 0 to 1-m stratum in Little Beaver Lake. Asterisks (*) in g-i) indicate non-detectable microcystin-LR ($<1 \mu\text{g g}^{-1}$ microcystin-LR). Microcystin-LR was quantified by HPLC.

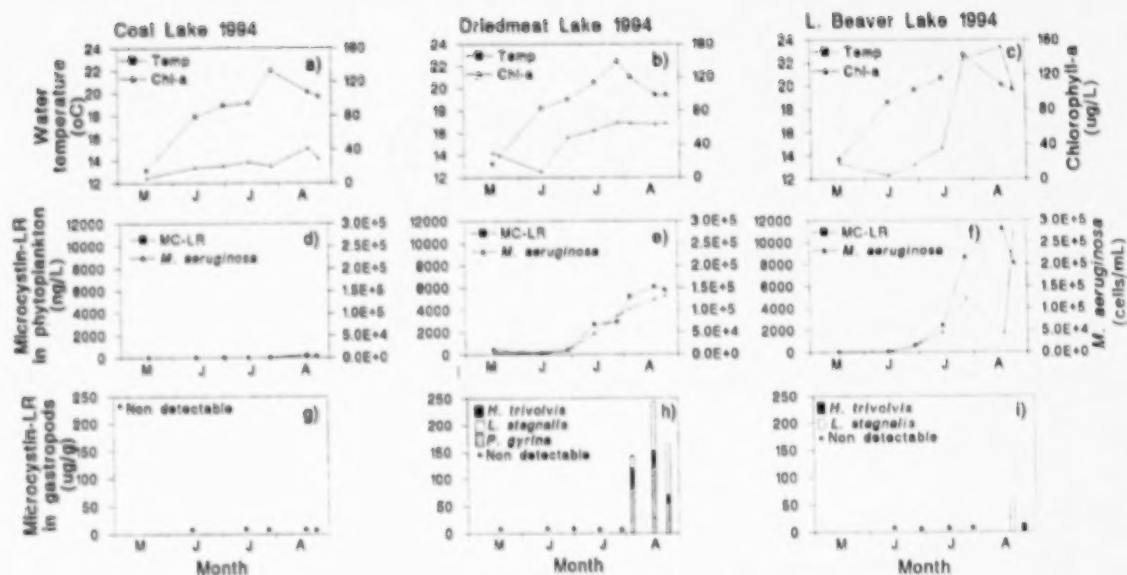


Figure 3. Seasonal changes in a-c) surface water temperature and chlorophyll *a*, d-f) *Microcystis aeruginosa* abundance and microcystin-LR concentration in the phytoplankton, and g-i) microcystin-LR concentration in three gastropod species for Coal (left panel), Driedmeat (middle panel), and Little Beaver (right panel) lakes for 1994. Except for gastropods, samples were collected from the 0 to 2-m stratum of the water column in Coal and Driedmeat lakes, and from the 0 to 1-m stratum in Little Beaver Lake. Asterisks (*) in g-i) indicates non-detectable microcystin-LR ($<1 \mu\text{g g}^{-1}$ microcystin-LR) in all three gastropod species. Microcystin-LR was quantified by HPLC.

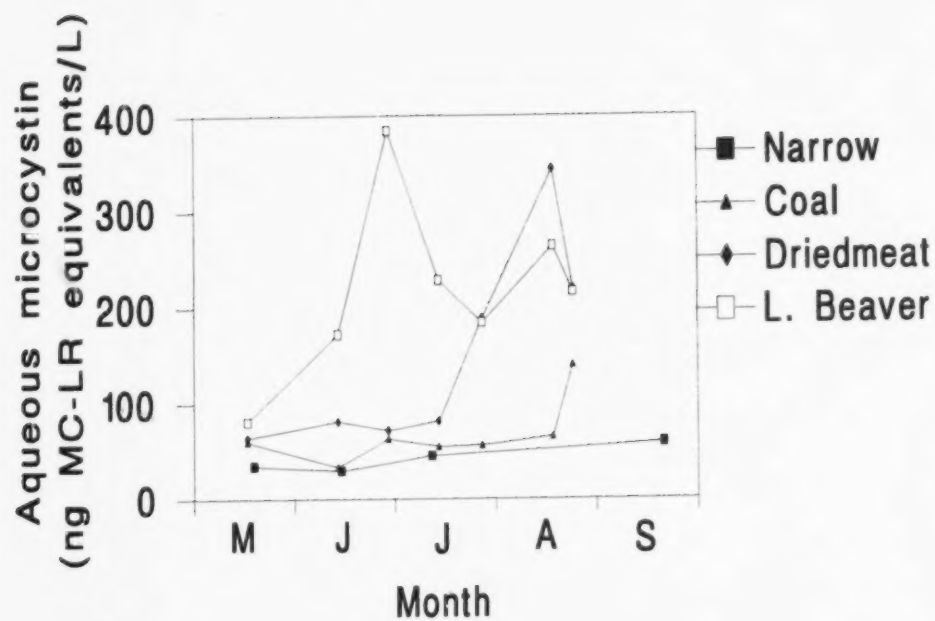


Figure 4. Seasonal changes in aqueous concentration of total microcystin in the upper 2 m of the water column (Coal, Driedmeat and Narrow lakes) or the upper 1 m of the water column (Little Beaver Lake) during 1994. Total microcystin concentration was determined by protein phosphatase assay and is expressed as microcystin-LR equivalent units per L.

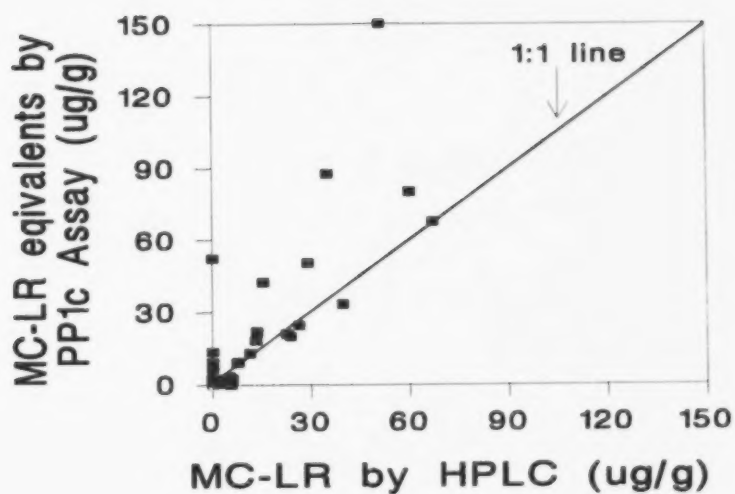


Figure 5. Comparison of microcystin-LR concentration quantified by HPLC and total microcystin concentration quantified by protein phosphatase assay for zooplankton samples. Total microcystin concentration is expressed as microcystin-LR equivalents.

a) Driedmeat Lake phytoplankton -
17 Aug 1994

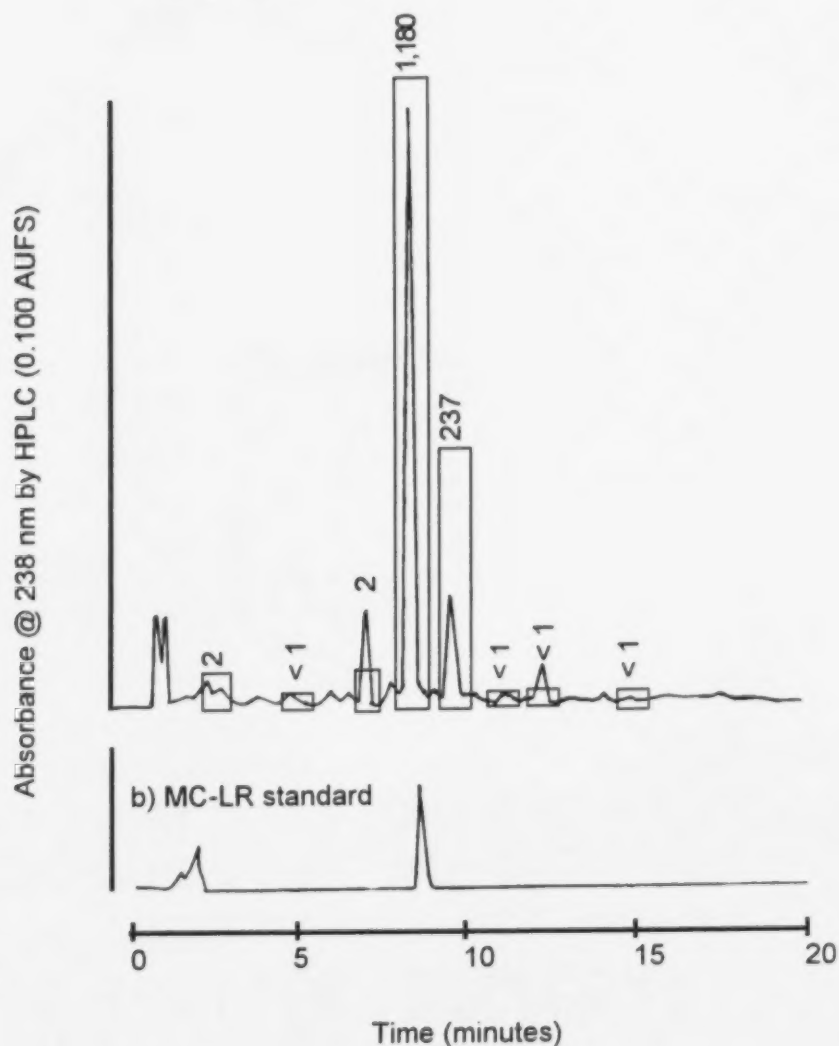


Figure 6. HPLC chromatogram of a) phytoplankton collected from Driedmeat Lake on 17 August 1994 and b) microcystin-LR standard. Analysis was on a Waters LC 1 HPLC with UV detection @ 238 nm. The sample and standard were run under linear gradient conditions (20 to 30% acetonitrile in 10mM ammonium acetate in 10 min, then 20% from 10 min to the end of the run [30 min]). Boxes in a) indicate fractions collected for protein phosphatase inhibition. Values above the boxes indicate microcystin concentration of the fraction, as quantified by protein phosphatase assay, and are expressed as ng microcystin-LR equivalents mL⁻¹. Values < 1 ng mL⁻¹ are considered non-detectable.

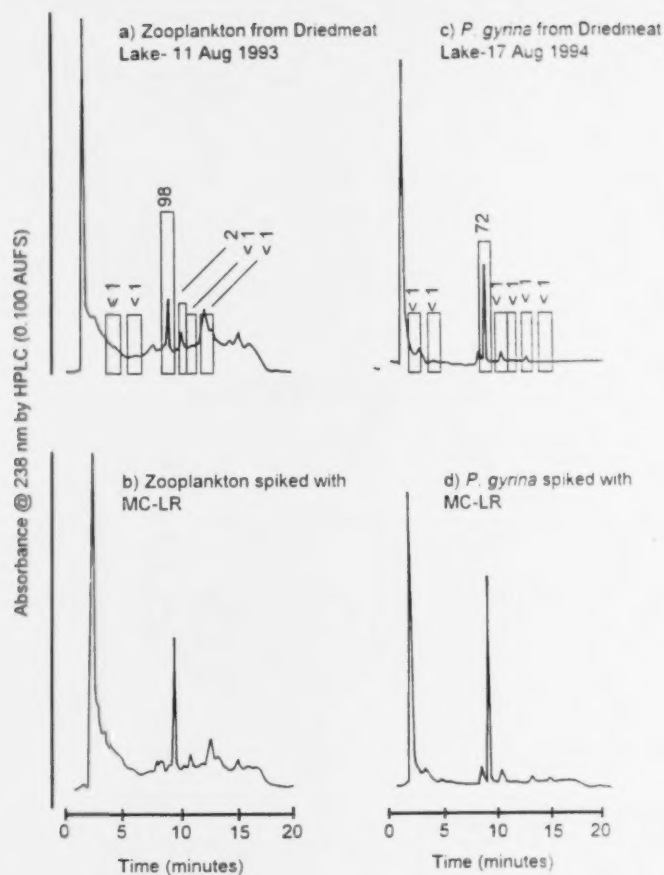


Figure 7. HPLC chromatogram of a) zooplankton collected from Driedmeat Lake on 11 August 1993, b) the above zooplankton sample spiked with microcystin-LR standard, c) *Physa gyrina* (gastropod) sample collected from Driedmeat Lake on 17 August 1994, and d) the above gastropod sample spiked with microcystin-LR. Analysis was on a Waters LC I HPLC with UV detection @ 238 nm. The sample and standard were run under linear gradient conditions (20 to 30% acetonitrile in 10mM ammonium acetate in 10 min, then 20% from 10 min to the end of the run [30 min]). Boxes in a) +c) indicate fractions collected for protein phosphatase inhibition. Values above the boxes indicate microcystin concentration of the fraction, as quantified by protein phosphatase assay, and are expressed as ng microcystin-LR equivalents mL⁻¹. Values < 1 ng mL⁻¹ are considered non-detectable.

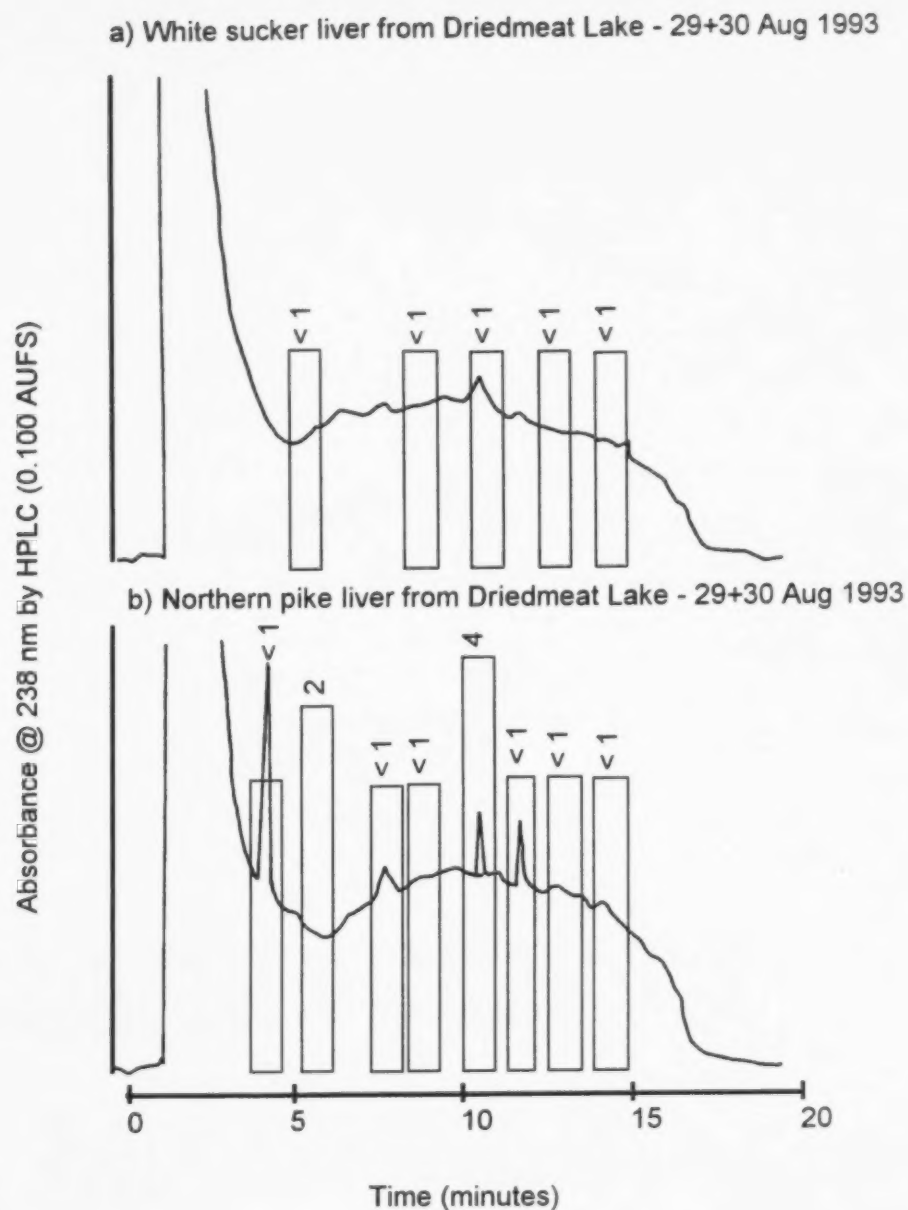


Figure 8. HPLC chromatogram of a) liver extracted from one white sucker collected from Driedmeat Lake in August 1993 and b) liver of a northern pike collected from Driedmeat Lake in August 1993. Analysis was on a Waters LC I HPLC with UV detection @ 238 nm. The two samples were run under linear gradient conditions (20 to 30% acetonitrile in 10mM ammonium acetate in 10 min, then 20% from 10 min to the end of the run [30 min]). Boxes indicate fractions collected for protein phosphatase inhibition. Values above the boxes indicate microcystin concentration of the fraction, as quantified by protein phosphatase assay, and are expressed as ng microcystin-LR equivalents mL^{-1} . Values $< 1 \text{ ng mL}^{-1}$ are considered non-detectable.

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Chapter 7

Comparative pathology of intraperitoneally-administered microcystin-LR in mice (*Mus musculus*) and rainbow trout (*Oncorhynchus mykiss*)⁵

Introduction

Hepatotoxins, produced by cyanobacteria in eutrophic lakes and ponds have been linked with poisonings and the deaths of livestock, wildlife and pets (Schwimmer and Schwimmer 1968; Carmichael and Gorham 1978; Galey et al. 1987). Microcystin-LR (MC-LR) is one of more than 50 widely occurring hepatotoxic cyclic peptides that are produced primarily by the cyanobacterium *Microcystis* spp. (Carmichael 1994; Rinehart et al. 1994) and its occurrence and concentration in phytoplankton can be variable in lakes both temporally (within and between years, and over 24 h.) and spatially (within and between lakes) (Kotak et al. 1995). Accidental ingestion of microcystin-containing water or cells by mammals, or intraperitoneal injection into laboratory rodents can cause massive hemorrhage in the liver and death (Schwimmer and Schwimmer 1968; Kotak et al. 1993b).

Available studies on the pathology of cyanobacterial hepatotoxins have focused on mammals, particularly laboratory rodents (Falconer et al. 1981; Dabholkar and Carmichael 1987; Adams et al. 1988; Hooser et al. 1989; Miura et al. 1989; Pace et al. 1991).

Although suspected as a possible cause or contributing factor to summerkill of fish in eutrophic lakes, there is no direct link between fish kills and microcystins. For example, Rodger et al. (1994) reported liver lesions in moribund brown trout collected from Loch Leven, Scotland, during an extensive bloom of microcystin-producing *Anabaena flos-aquae*. Although the liver lesions were similar to those reported for mammals exposed to microcystins, the link between the toxin-producing phytoplankton, liver lesions and fish mortalities remained speculative. Very little information exists on the pathology of

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cyanobacterial hepatotoxins in fish (Phillips et al. 1985; Sugaya et al. 1990; Rabergh et al. 1991; Andersen et al. 1993; Tencalla et al. 1994), despite the common aquatic environment. Based on the preceding studies, microcystins appear to be less acutely toxic to fish than to mice, although there is a large degree of variation within and between species of fish with respect to the dose-mortality response. In addition, the pathological effects of microcystins in mammals and fish may be quite different. This study compares the acute hepatic and renal toxicity of intraperitoneally-administered MC-LR to rainbow trout and laboratory mice.

Materials and Methods

Microcystin-LR - Microcystin-LR (MC-LR, >95% pure based on HPLC area counts) was obtained by purification of a cyanobacterial extract with reverse phase HPLC (Kenefick et al. 1992). Briefly, 60 g (dry weight) of cyanobacteria collected from Driedmeat Lake, Alberta on 25 August 1992 was extracted twice for 24 h in 5% acetic acid. The extract was vortexed and then centrifuged to remove cellular material. The extract was filtered through a Whatman GF/C filter and passed through preconditioned Supelco LC-18 disposable cartridges. Microcystins were eluted from the cartridge in methanol and MC-LR was isolated and purified by HPLC (Waters 6000A pumps, HP 1040A diode array detector @ 238 nm, Waters μ -Bondapak semipreparative column, isocratic conditions at 76:24 [10 mM ammonium acetate: acetonitrile]). MC-LR was dissolved in 0.9% NaCl immediately prior to use.

Animals - Rainbow trout (*Oncorhynchus mykiss*) (79-151 g) were obtained from Sun Valley Trout Farm, Mission B.C. The fish were held in fibreglass circular 680 L fish rearing tanks containing dechlorinated municipal water, recirculated at 3 L min⁻¹. The fish were maintained at 15 °C and were fed commercial trout chow (Martin Feed Mills, Elmira and Tavistock, ON.) at 3% of body weight d⁻¹. Both fish and mice were maintained according to standard husbandry procedures (Canadian Council of Animal Care 1984). Male CD-1 mice (*Mus musculus*) (20-25 g) were obtained from Charles River Canada, Inc., St. Constant, Quebec and groups of 5 mice were housed in shoebox cages. The mice were fed Purina Certified Laboratory Rodent Chow (Ralston Purina Co., Richmond, IN.)

ad libitum. Tap water in bottles was available *ad libitum* and was changed every 3 days. Fish and mice were allowed to acclimate for 14 days prior to experimentation. Food was withheld 48 h (fish), and 24 h (mice) prior to experimentation and throughout the duration of the experiment.

Fish were weighed in a vessel containing water and randomly assigned to three treatment groups. With 1 cm, 22 gauge needles, doses were injected intraperitoneally along the ventral midline, approximately 1 cm anterior to the pelvic fin at the following doses: two fish (controls) received 0 $\mu\text{g kg}^{-1}$ MC-LR (0.5 mL, 0.9% NaCl), six fish were injected with 400 $\mu\text{g kg}^{-1}$ MC-LR and four fish received 1,000 $\mu\text{g kg}^{-1}$ MC-LR. Volumes of injections ranged from 0.1 to 0.6 mL. Fish were continuously monitored for changes in behavior, swimming and coloration.

Mice were weighed and randomly assigned to four treatment groups which were given intraperitoneal injections at the following doses: two mice (controls) received 0 $\mu\text{g kg}^{-1}$ MC-LR (0.5 mL, 0.9% NaCl), four mice received 25 $\mu\text{g kg}^{-1}$ MC-LR, ten mice received 50 $\mu\text{g kg}^{-1}$ MC-LR and five mice received 100 $\mu\text{g kg}^{-1}$ MC-LR. The mice were observed continuously for the first 5 h and then every 2 to 4 h thereafter. Observations included changes in behavior and the coloration of the eyes, ear and tail.

Surviving animals were euthanized 24 to 26 h (mice) or 26 h (fish) after treatment. Moribund fish and those surviving at the termination of the experiment were sacrificed by a sharp blow to the head, while moribund mice and those that survived to the termination of the experiment were anaesthetized with carbon dioxide and then sacrificed by exanguination via posterior vena cava severance.

All animals were weighed, a complete necropsy examination was performed, and gross lesions of internal organs were recorded. The livers were excised, freed of attached tissue, then weighed and mass was expressed as a percentage of the body mass. Kidneys in the mice were also weighed.

Histopathology and Ultrastructure - Sections (5 mm) of liver and kidney (anterior and posterior) of fish were fixed in Bouin's fixative. Sections of similar tissues from mice were fixed in 10% neutral buffered formalin. The fixed tissues were processed routinely,

embedded in parafin, cut at 4-6 μm thickness, stained with hematoxylin and eosin (HE), and examined by light microscopy (LM).

Tissues were prepared for electron microscopic examination according to the method of Bozzolla and Russell (1992). Briefly, thin sections (0.5 to 1.0 μm) of liver and kidney were fixed for 24 h at 4 °C in a mixture of 1.5% glutaraldehyde and 1.0% formaldehyde in 0.1M phosphate buffer (pH 7.4). The fixed tissues were post-fixed in 1.0% osmium tetroxide in 0.1M phosphate buffer (pH 7.2) for 1 h. Tissues were rinsed in the same buffer and then in distilled water, "en block" stained with 2% uranyl acetate, dehydrated in a graded series of ethanol, and embedded in Spurr resin. Semithin sections (1 μm) were cut, stained with toluidine blue and examined by LM, and appropriate sites containing visible lesions were selected for further sectioning for EM. Ultrathin sections (90-100 nm) of selected sites were cut, mounted on copper grids, stained with 4% uranyl acetate and Reynolds lead citrate and examined on an H-6000 electron microscope (Hitachi Ltd., Tokyo, Japan).

Statistical Analysis - The mean percent of liver to body mass ratio was computed for all experimental groups. These values were compared between treatment and control groups, by Student *t*-test with SPSS for Windows 6.0 software (SPSS Inc., Chicago, IL.). The level of significant difference was set at 0.05.

Results

Clinical Observations - All mice (treatment and control) became lethargic immediately after injection but resumed normal activity within five minutes. Control fish similarly became slightly lethargic following injection but resumed normal swimming activity approximately 1 h after injection. In contrast, fish injected with microcystin-LR (MC-LR) remained lethargic for extended periods, interrupted by quick bursts of frenetic swimming. The depression and lethargy became more pronounced with time.

Fish injected with MC-LR underwent a color change (of the dorsum) from light silvery-green to a dark olive-green. Treated fish also lost swimming coordination and buoyancy control which increased with time and was most severe in the 1,000 $\mu\text{g kg}^{-1}$ dose group. Fish in this group frequently floated near the surface of the water, either on

their side or upright with the head facing down. Also in this dose group there was yellowing, or jaundice, of the connective tissue around the eyes, the membranous parts of the fins, and the underside of the body. Terminally, opercular ventilatory movements gradually decreased in frequency and magnitude. Fish in the 400 $\mu\text{g kg}^{-1}$ dose group also exhibited sluggish swimming and loss of buoyancy control, but to a lesser degree.

Mice injected with MC-LR began showing signs of distress 30 min after treatment. Signs included huddling, ruffling of the fur, lethargy interrupted by sudden bursts of leaping, and incoordination. Periods of lethargy in the 50 and 100 $\mu\text{g kg}^{-1}$ dose mice lengthened gradually until the animals lost the ability to move. Breathing became very shallow and rapid until the mice were near death, when it became very slow. Marked pallor of the eyes, ears and tail became more pronounced with time, and was particularly evident in the two highest dose groups. Clinical signs were not observed in the control mice.

Mortality was observed in 9/10 and 5/5 mice in the 50 and 100 $\mu\text{g kg}^{-1}$ dose groups respectively, but not in the 25 $\mu\text{g kg}^{-1}$ dose group or controls. All four (4/4) fish in the 1,000 $\mu\text{g kg}^{-1}$ dose group died (5, 10, 18 and 25 h after treatment) but there was no mortality in the 400 $\mu\text{g kg}^{-1}$ dose group or the controls over the 26-h period.

Gross Pathology - Gross changes in microcystin-treated fish were present primarily in the livers. Fish in the 1,000 $\mu\text{g kg}^{-1}$ dose group had distinct yellowish discoloration of the membranous parts of all fins, the connective tissue around the eyes, peritoneum and fat of the body cavity, and loose connective tissue between the muscle. The body cavity typically contained 7 to 10 mL of straw-colored or blood-tinged fluid, which appeared to ooze from the liver. Livers were wet, soft and pale with multiple, small ecchymoses visible beneath the capsule. Fish in the 400 $\mu\text{g kg}^{-1}$ dose group had red/tan mottled livers. Bile within the gall bladders of all microcystin-treated fish appeared greenish yellow in contrast to the light yellow bile of the control fish.

Gross changes in the microcystin-treated mice were also present in the livers, however, all somatic tissues were pale and devoid of blood. In contrast to the pale livers of microcystin-treated fish, livers from microcystin-treated mice were dark red, moderately

enlarged and friable. A mottled appearance was present (most prominent in the central lobule) of 1/4 mice in the 25 $\mu\text{g kg}^{-1}$ dose group, in 9/10 mice in the 50 $\mu\text{g kg}^{-1}$ dose group and in all mice from the 100 $\mu\text{g kg}^{-1}$ dose group.

The liver mass to body mass ratio (Table 1) increased in both microcystin-treated fish and mice as compared to the controls. In fish, the ratio was significantly higher in the 400 $\mu\text{g kg}^{-1}$ dose group compared to the control group ($P=0.0004$, t -test). Although the average liver to body mass ratio in the 1,000 $\mu\text{g kg}^{-1}$ dose group was much higher than that of the control group (Table 1), the ratios were not significantly different due to the high variation ($\text{CV}=43\%$) and small sample size. The liver to body mass ratio of mice from all three dose groups were significantly higher than that of control mice (Table 2). These ratios increased with increasing dose. Kidney mass to body mass ratios were not different in treated versus control mice.

Histopathology of the Liver - The severity of hepatocellular degenerative changes in the livers of microcystin-treated fish and mice was both time and dose-dependent. Fish in the 1,000 $\mu\text{g kg}^{-1}$ dose group (which died within 10 h following treatment), and those in the 400 $\mu\text{g kg}^{-1}$ dose group (killed at 26 h) had diffusely swollen and vacuolated hepatocytes (Fig. 1b) which obliterated sinusoidal spaces. Multiple, variably-sized foci of centrilobular, midzonal or bridging coagulative necrosis were present (Fig. 1c). The temporal sequence of liver damage appeared to progress as follows: swelling/vacuolation of periportal hepatocytes, more severe swelling with nuclear pyknosis in midzonal hepatocytes, and coagulative necrosis of hepatocytes surrounding the central veins. Fish in the 1,000 $\mu\text{g kg}^{-1}$ dose group (which died 18 h or more after treatment) exhibited diffuse, coagulative hepatocellular necrosis. Additionally, there were foci of liquifactive hepatocellular necrosis (disruption of cell membranes and lysis, producing a milieu of cell debris) involving entire lobules (i.e., massive). In such areas, often the only residual cells were short chords of attenuated endplate hepatocytes or proliferative bile ducts. Zones of necrotic hepatocytes were also present subjacent to Glisson's capsule. Rarely, there was mild hemorrhage in areas of extensive hepatocellular loss. Sloughed hepatocytes occasionally appeared within central veins. Livers from control fish displayed no such lesions (Fig. 1a).

In contrast to fish, the primary lesion in the microcystin-treated mice was severe hepatocellular loss and hemorrhage. In mice from the 100 $\mu\text{g kg}^{-1}$ dose group there was diffuse, marked centrilobular to midzonal congestion and hemorrhage (Fig. 1e). In these areas, zones of hepatocellular loss were filled by blood which compressed peripheral residual hepatocytes. Extensive zones of hemorrhage were also present subjacent to Glisson's capsule. In contrast to fish, residual centrilobular hepatocytes were typically dissociated from one another (Fig. 1f) and were rounded. Cell membranes in mice were intact (Fig. 1f) unlike the frequently lysed hepatocytes present in the livers of microcystin-treated fish. Additionally, there was diffuse, moderate periportal and bridging hepatocellular vacuolar change. Occasionally, sloughed hepatocytes were present within intrahepatic veins.

In mice from the 50 $\mu\text{g kg}^{-1}$ dose group, centrilobular to midzonal congestion and hemorrhage were slightly less severe, were typically multifocal rather than diffuse, and contained more dissociated hepatocytes than in livers from the 100 $\mu\text{g kg}^{-1}$ dose group. Vacuolar change of the periportal hepatocytes was also less severe. In one mouse, there was only a single focus of hepatocellular necrosis. This mouse survived the 24 h. In the 25 $\mu\text{g kg}^{-1}$ dose group, lesions were present in only one mouse which also had gross changes. Histopathologic changes in this mouse were similar to those described for mice in the 100 $\mu\text{g kg}^{-1}$ dose group. No microscopic changes were detected in the livers of control mice (Fig. 1d).

Ultrastructure of Liver - Hepatocytes were the principal cell type affected by MC-LR in both fish and mice. Hepatocytes from fish in the 1,000 $\mu\text{g kg}^{-1}$ dose group had marked karyorrhexis, diffuse dilation of cisternae of the endoplasmic reticulum (from which ribosomes had been shed), and overall swelling/rounding up of the cells (Fig. 2b +a, respectively). There was also widespread disruption of hepatocyte cell membranes, resulting in extensive zones of cell debris (Fig. 2b). Numerous electronlucent, membrane-bound vacuoles (Fig. 2b, inset) were evident in necrotic hepatocytes. Hepatocellular changes in fish from the 400 $\mu\text{g kg}^{-1}$ dose group were similar but less severe than those in the 1,000 $\mu\text{g kg}^{-1}$ dose group. In contrast to microcystin-treated fish, hepatocytes from

control fish were irregular in shape, had well-defined plasma membranes and lacked electronlucent vacuoles (Fig. 2a).

Hepatocytes from microcystin-treated mice were similar to those described in fish. In contrast to the hepatocellular lysis present in fish, however, in mice the integrity of the cell membranes was almost always maintained. Also in mice, unlike fish, there was widespread hemorrhage between hepatocytes resulting in loss of cell adhesion (not shown).

Histology and Ultrastructure of Kidney - Histopathologic changes were present only in the posterior kidney from the two longest surviving fish in the $1,000 \mu\text{g kg}^{-1}$ dose group. Changes consisted of coagulative necrosis of the entire tubular epithelium in some tubules, while other tubules were lined by degenerated cells. Cell debris and even cellular casts variably filled tubular lumina (Fig. 3b). Affected tubules were present admixed with regenerative or normal tubules (Fig. 3b). No changes were present in control fish (Fig. 3a).

Ultrastructural changes in the kidneys of the two fish with histopathologic lesions consisted of tubules lined predominantly by light-staining cells. These cells contained several electronlucent or variably-dense membrane-bound vacuoles, such as those described in the livers of microcystin-treated fish. In the most severely affected tubules, there was necrosis and lysis of the entire epithelial lining resulting in an intact basal lamina encircling a zone of cellular debris (Fig. 3d). These tubules were greatly expanded in comparison to tubules from control fish (Fig. 3d+c, respectively). No changes were present in the renal tubular epithelial cells in control fish (Fig. 3c), in any other microcystin-treated fish, or in mice.

Discussion

Rainbow trout appeared more tolerant of high microcystin-LR (MC-LR) doses than mice (Table 3). The lowest dose administered to the fish, which did not cause any mortality within 26 h, was four times higher than the dose causing 100% mortality in the mice over the same time period. The higher tolerance and longer survival of the fish (up to 24 h vs 1-2 h for the mice) may be explained by the slower perfusion rate of blood

through the liver in the fish (less than one-quarter that of mammals, Gingerich 1982), which may reduce the initial dose reaching the liver in the fish compared to a mouse. In addition, the longer survival and higher dose tolerance to microcystins shown by fish may also be due to slower uptake kinetics of the toxin by the fish hepatocytes, although this has not been determined. Runnegar et al. (1991) found that the uptake of microcystin-YM by isolated rat hepatocytes was dramatically reduced by decreasing ambient incubation temperature. Since most fish exist in an environment that is much colder than the mammalian core body temperature (37 °C), a slower uptake of microcystins by fish hepatocytes based only on ambient temperature could explain the longer survival and higher dose tolerance of fish compared to mice. In addition, fish hepatocytes may also have different uptake kinetics than mammalian hepatocytes at equivalent temperatures. This also has not been studied. Alternatively, the higher tolerance and survival of the fish may be explained by the coevolution of fish with toxin-producing phytoplankton in productive lakes, although this hypothesis has never been tested. Fish may have evolved one or several mechanisms to detoxify these natural toxins. Because mice would not have had a similar close association with toxic algae, they could be more susceptible.

While the LD₅₀ of MC-LR in mice (by intraperitoneal injection) is approximately 50 µg kg⁻¹ (Carmichael 1988), the LD₅₀ in fish appears to be higher. For example, Rabergh et al. (1991) found that the LD₅₀ of MC-LR in common carp (*Cyprinus carpio*) by intraperitoneal (i.p.) injection was between 300 and 550 µg kg⁻¹ over 7 d. Andersen et al. (1993) reported few mortalities in Atlantic salmon (*Salmo salar*) injected i.p. three times with MC-LR at 555 µg kg⁻¹ injection⁻¹ (spaced three days apart) over a 36 d experiment. In contrast, Phillips et al. (1985) recorded 100% mortality in rainbow trout injected i.p. with a *Microcystis aeruginosa* extract equivalent to the i.p. LD₅₀ in mice. Therefore there may be considerable differences in the susceptibility to MC-LR between species of fish. In addition, Rabergh et al. (1991) demonstrated that there was variation within a species (carp), with the LD₅₀ ranging from 80 to between 300 and 550 µg kg⁻¹ with two batches of fish. The results of the present study suggested that the LD₅₀ of MC-LR in rainbow trout was between 400 and 1,000 µg kg⁻¹, comparable to the results of

Tencalla et al. (1994) in which there was 100% mortality of rainbow trout within 24 h after i.p. injection with a dose of $550 \mu\text{g kg}^{-1}$ MC-LR.

The liver was the organ most profoundly affected by MC-LR in both rainbow trout and mice in this study (Fig. 1+2). However, the mechanism(s) of toxicity appear to be quite different. For example, MC-LR causes massive hemorrhage of the liver in mammals and a resultant increase in the liver to body mass ratio, as much of the systemic blood ends up pooling in the liver (Adams et al. 1988; Kotak et al. 1993a+b). The livers from microcystin-exposed mice in this study had marked hemorrhages and were dark red. Consequently, the liver to body mass ratio was significantly higher than in control mice because of this influx of blood (Table 2). In contrast, livers from the rainbow trout in the present study ranged from dark red (saline controls) to pale tan, and even to a creamy-white in a few individuals at the highest dose. The change in color may reflect the decreasing ability of the liver to process bile pigments as liver damage progressed. The membranes surrounding the eyes and operculum, fins and muscle of some fish in the highest dose group appeared yellow or jaundiced, supporting this hypothesis (Table 3). Although the liver to body mass ratio also increased in the fish (Table 1), hemorrhage was never a prominent feature in the fish. The increase in liver weight in the fish may have been caused by hepatocellular swelling (i.e., hydropic degeneration) by cells since straw-colored fluid oozed from the fish livers when they were sectioned. The lack of hemorrhage may be partially due to the lower blood flow through the fish liver in comparison to mice. I therefore believe that the ultimate cause of death in fish may be due to general hepatic failure caused by massive hepatocyte necrosis rather than hypovolumic shock, as was reported for mice (Carmichael 1992). Rabergh et al. (1991) reached a similar conclusion concerning the effects of MC-LR on carp.

Microcystin-LR caused substantial changes in the liver in both fish and mice (Table 3, Fig. 1+2). Liver damage, seen by light microscopy, was the most severe in the highest dose groups. Diffuse hydropic degeneration imparting hepatocellular hypertrophy were observed in both fish and mice, but hepatocyte dissociation was evident only in mice (Fig. 1c+f). Intact hepatocytes were commonly found pooled in the central vein in the mice, while they were only occasionally observed in fish. In livers of MC-LR exposed fish,

multifocal to massive zones of liquifactive hepatocyte necrosis were found next to islands of seemingly intact cells. Hemorrhage in the fish livers was only mild. The highest MC-LR dose resulted in complete loss of liver architecture in the fish (Fig. 1c). Histological lesions in the mice however, consisted of multifocal to massive lobular hemorrhage. Unlike the progression of histological lesions in rats and mice, which are reported to start centrilobularly (Hooser et al. 1989, 1990), the progression of lesions in the fish livers did not display any pattern of progression, perhaps because of the lack of distinct zonation of the fish liver. Hepatocellular damage was usually massive and not restricted to a particular region of the liver lobule (e.g., centrilobular, periportal, midzonal).

Histological lesions in the fish livers caused by i.p. exposure to MC-LR, including severe hydropic degeneration and diffuse hepatocyte necrosis, were consistent with lesions reported by Kent et al. (1988) and Kent (1990) in net-pen reared Atlantic salmon afflicted with net-pen liver disease (NLD) along the coast of British Columbia and the State of Washington. In the case of salmon, liver lesions were suspected to be caused by ingestion of biota containing MC-LR, which were fouling the net-pens (Andersen et al. 1993). However, in salmon injected i.p. with purified MC-LR, megalocytosis was also observed in the fish livers several days after exposure and increased in frequency with exposure time. This type of lesion was not evident in the fish used in the present study, likely reflecting the short exposure period (26 h) compared to the longer exposure in the study by Andersen et al. (1993) (up to 36 d).

Ultrastructural changes in the liver in fish and mice differed primarily in the status of the hepatocyte plasma membranes. Rounded, intact hepatocytes were commonly adjacent to necrotic ones in which the plasma membrane was completely disrupted (Fig. 2b). Cellular organelles thus were random within the cytosol, outlined by only the remnants of a plasma membrane (Fig. 2b; Table 3). This contrasted sharply with the ultrastructural changes in the mouse liver in which cell membranes remained intact, although cell adhesion was not maintained. Hepatocytes of microcystin-treated fish and mice were rounded and contained many clear, coalescent vacuoles (Fig. 2b, inset). Some hepatocytes contained pyknotic and/or karyorrhectic nuclei. The rough endoplasmic reticulum (RER) in both fish and mice shed their ribosomes and were occasionally

whorled, and the mitochondria appeared swollen. There was a dilation of the space of Disse and a loss of microvilli. Additionally, there was massive subcapsular necrosis of hepatocytes along the outer perimeter of the liver in both the fish and mice in the present study, possibly because of absorption of the toxin directly across the capsule from the peritoneal cavity.

Many of the effects seen in the liver at the ultrastructure level such as swollen mitochondria and loss of ribosomes from RER are likely secondary in nature. The primary mode of action of microcystins is thought to be the inhibition of cellular enzymes, protein phosphatases (Eriksson et al. 1990; MacIntosh et al. 1990). These enzymes regulate the dephosphorylation of protein kinases and therefore are important in a wide range of cellular metabolic pathways including glycogen metabolism, cell division and the arrangement of cytoskeletal filaments. The inhibition of protein phosphatases 1 and 2a results in an increase in the phosphorylation state of protein kinases that is thought to cause a reorganization of the hepatocyte cytoskeletal elements, causing hepatocyte deformation (Runnegar et al. 1981; Eriksson et al. 1990; Hooser et al. 1991). The deformation of mammalian hepatocytes, caused by protein phosphatase inhibition, may contribute to massive hemorrhage in the liver. The lack of widespread hemorrhage in the fish liver, and destruction of hepatocyte plasma membranes suggests that mechanisms other than, or in combination with, protein phosphatase inhibition may be responsible for the pathological effect seen in the fish livers.

Although microcystins are described as hepatotoxins, this study indicates that kidney tubular epithelial cells in fish are also affected (Fig. 3). In some cases, disruption of renal tubular epithelial plasma membranes resulted in the formation of casts comprised of cellular debris within the tubular lumen. It appears that kidney tubular cells may possess a transport mechanism similar to that of the hepatocytes (the multispecific bile acid transport system) which is responsible for the uptake of the toxin into the cell (Runnegar et al. 1981; 1991). Rabergh et al. (1991) has also reported degenerative changes in the epithelial cells of the tubule, glomeruli and interstitial tissue of the kidney in carp exposed i.p. to MC-LR.

In contrast to fish, no renal damage was observed in the mice. The more prominent kidney damage seen in the fish compared to that observed in mice and rats (Hooser et al.

1989; Meriluoto et al. 1989) might be explained by the length of time the animals survive a lethal dose. Mice generally die within 1-2 h after i.p. injection of MC-LR (Kotak et al. 1993b). Under these circumstances, the toxin likely has its greatest impact on the liver. Fish survive a lethal insult of MC-LR much longer and as a result, the toxin remains in circulation much longer. This may allow the toxin to reach the kidney in much higher concentration than in the case of mice.

In summary, this study indicated that rainbow trout were markedly different from mice with respect to their dose tolerance, survival time, and resulting pathological effects when exposed to intraperitoneally-administered MC-LR. To my knowledge pathologic lesions including liquifactive hepatocyte necrosis and a lack of hemorrhage in the liver of fish exposed to MC-LR is a new finding. This study, as with most other studies examining the toxicity of microcystins to animals, focussed on acute exposure by intraperitoneal injection. Given the recent findings that microcystins are potent tumor promoting agents (Fujiki and Suganuma 1993), and that animals, including humans, may be exposed to chronic doses of microcystins in drinking water over a lifetime, more attention should be directed to examining sublethal and chronic health effects, particularly by focussing on an oral route of exposure. For example, Kotak et al. (submitted) have shown that aquatic invertebrates (e.g., gastropods, zooplankton), which are a food source for many fish, can accumulate microcystins. Therefore, fish may be exposed to microcystins through their food base for extended periods of time over the summer in productive lakes. Virtually nothing is known about the oral toxicity of microcystins to fish, and how chronic exposure may affect growth rates and reproduction. Because the role of microcystins in summer fish kills in productive lakes (speculated to be due to the uptake of dissolved microcystins via the gills after an algal bloom dies off) remains unresolved, an examination of the acute and chronic health effects in fish by oral exposure to microcystins needs to be addressed.

Table 1. The effect of intraperitoneally-administered microcystin-LR on mean liver to total body mass ratio (%) in rainbow trout. Fish in the saline and 400 $\mu\text{g kg}^{-1}$ treatment groups were sacrificed at 26 h post-injection while the four fish in the 1,000 $\mu\text{g kg}^{-1}$ treatment group died at 5, 10, 18 and 25 h post-injection. S.E.= Standard Error of the mean.

Microcystin-LR Dose ($\mu\text{g kg}^{-1}$)	Mean Liver/Body Mass (%)	S.E.
0 (saline)	0.80	0.00
400	1.31	0.04
1,000	1.45	0.31

Table 2. The effect of intraperitoneally-administered microcystin-LR on mean liver and kidney to total body mass ratios (%) in mice. Mice were injected with either saline or microcystin-LR in saline and were sacrificed either just prior to death (usually 1.5 h post-injection for mice given a lethal dose) or at 26 h post-injection in those mice surviving. S.E. = Standard Error of the mean.

Microcystin-LR Dose ($\mu\text{g kg}^{-1}$)	Mean Liver/Body Mass (%)	S.E.	Mean Kidney/Body Mass (%)	S.E.
0 (saline)	5.3	0.6	1.79	0.07
25	6.7	0.1	1.87	0.08
50	9.6	0.5	2.03	0.06
100	10.2	0.2	1.92	0.08

Table 3. Comparison of the toxicity and pathology of intraperitoneally-administered microcystin-LR in mice and rainbow trout.

Feature	Mice	Rainbow Trout
Dose tolerance	no mortality at 25 $\mu\text{g kg}^{-1}$ 90% mortality at 50 $\mu\text{g kg}^{-1}$ 100% mortality at 100 $\mu\text{g kg}^{-1}$	No mortality at 400 $\mu\text{g kg}^{-1}$ 100% mortality at 1,000 $\mu\text{g kg}^{-1}$
Mean time to death	1.5 h	14.5 h
Clinical signs	lethargy, palor of eyes, ears and tail	lethargy, dark color dorsally, jaundice of mucus membranes and skin
Liver to body mass ratio	increased	increased
Gross Lesions: Liver	severe hemorrhage, swelling, no edema, no ascites, no jaundice	mild hemorrhage, enlargement, severe edema, severe ascites, severe jaundice
Kidney	no gross lesions	no gross lesions

Table 3. (continued)

Feature	Mice	Rainbow Trout
Histopathology: Liver	cell swelling, loss of cell adhesion, focal to massive hemorrhage, mild coagulative necrosis, embolic hepatocytes in many central veins	cell swelling, cell adhesion maintained, mild focal hemorrhage, severe multifocal to massive liquifactive hepatocyte necrosis, occasional embolic hepatocytes in central vein
Kidney	no histopathologic change	multifocal, coagulative tubular necrosis, dilation of Bowman's space
Ultrastructure: Liver	cell membranes intact, loss of cell adhesion, dense cytoplasmic vacuoles, vesiculation of ER	lysis of plasma membrane, maintenance of cell adhesion, clear vacuoles in cytoplasm, vesiculation of ER
Kidney	no ultrastructural changes	tubular epithelial necrosis, vacuoles in cytoplasm

Figure 1. Light microscopy of livers of a) control fish, b) fish in the $400 \mu\text{g kg}^{-1}$ dose group showing swollen hepatocytes and obliteration of sinusoidal spaces, c) fish in the $1,000 \mu\text{g kg}^{-1}$ dose group which died within 10 h, showing swollen hepatocytes, pyknotic nuclei and marked coagulative necrosis, and d) control mice, e) mice in the $100 \mu\text{g kg}^{-1}$ dose group showing a focus of marked hemorrhage, and f) mice in the $100 \mu\text{g kg}^{-1}$ dose group showing separation of intact hepatocytes amidst a focus of marked hemorrhage. Bars equal $50 \mu\text{m}$.

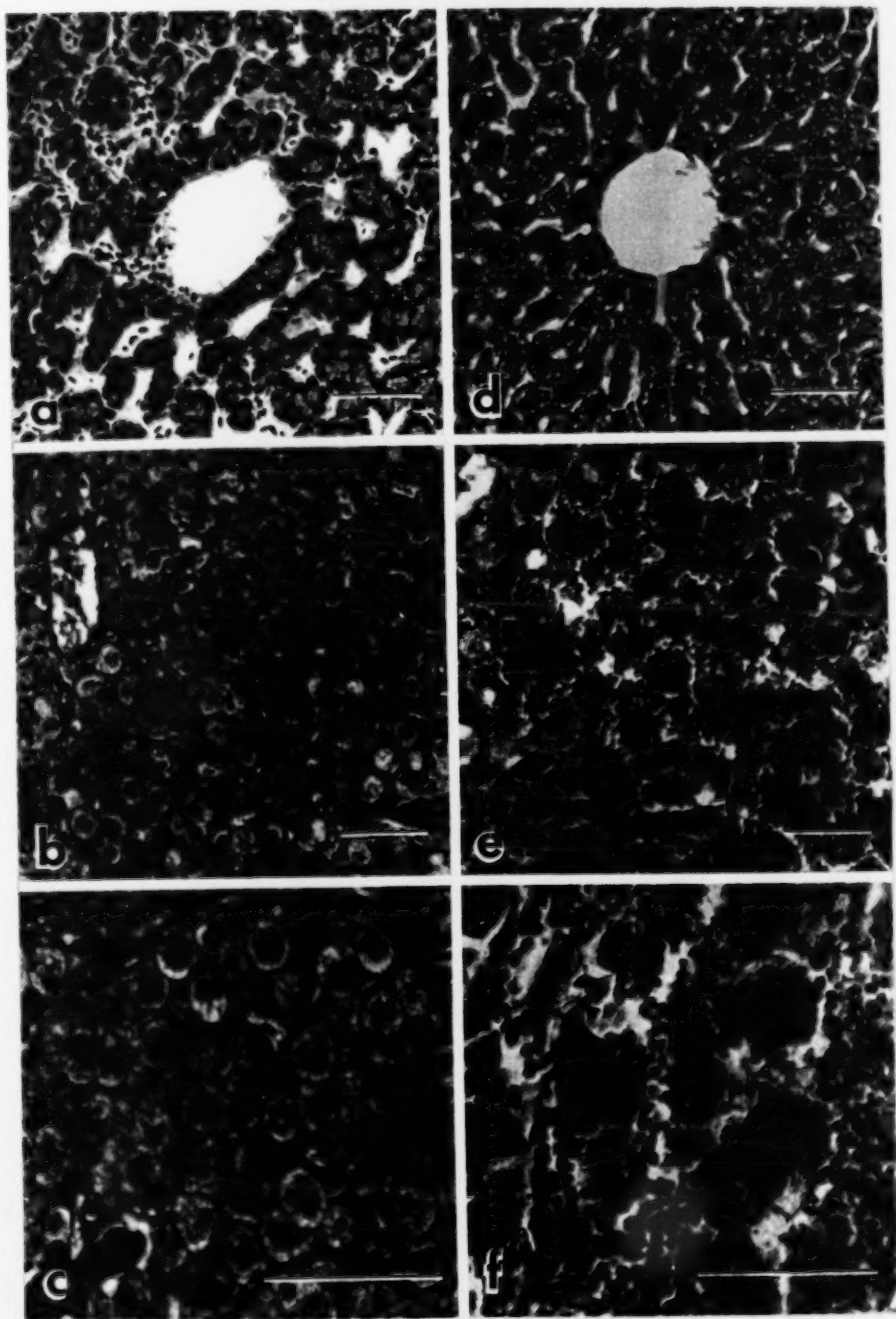


Figure 2. Electron micrograph of livers from a) control fish showing well-defined, irregular-shaped hepatocytes with profiles of RER, and b) a single, intact but degenerative, swollen and vacuolated hepatocyte from a fish in the $1,000 \mu\text{g kg}^{-1}$ dose group which died within 10 h. The hepatocyte is located within a milieu of hepatocellular debris. Inset is a focus of liquifactive hepatocyte necrosis containing numerous electronlucent vacuoles. Bars equal $1.0 \mu\text{m}$.

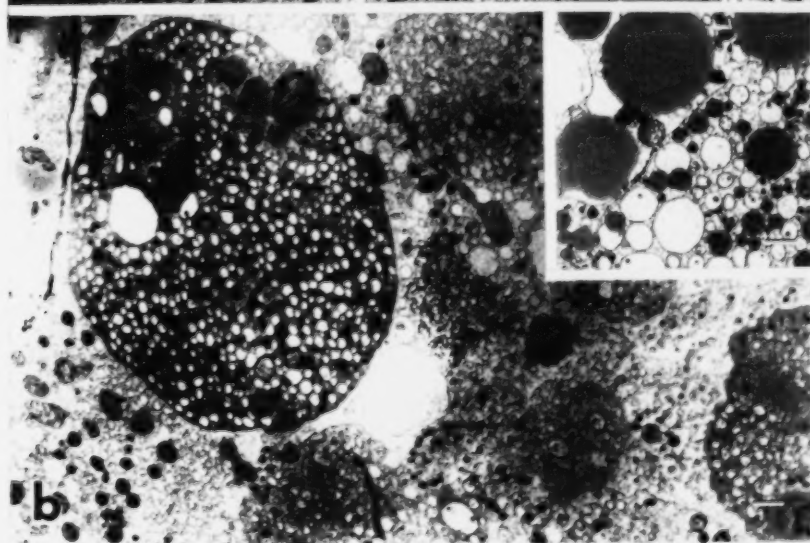
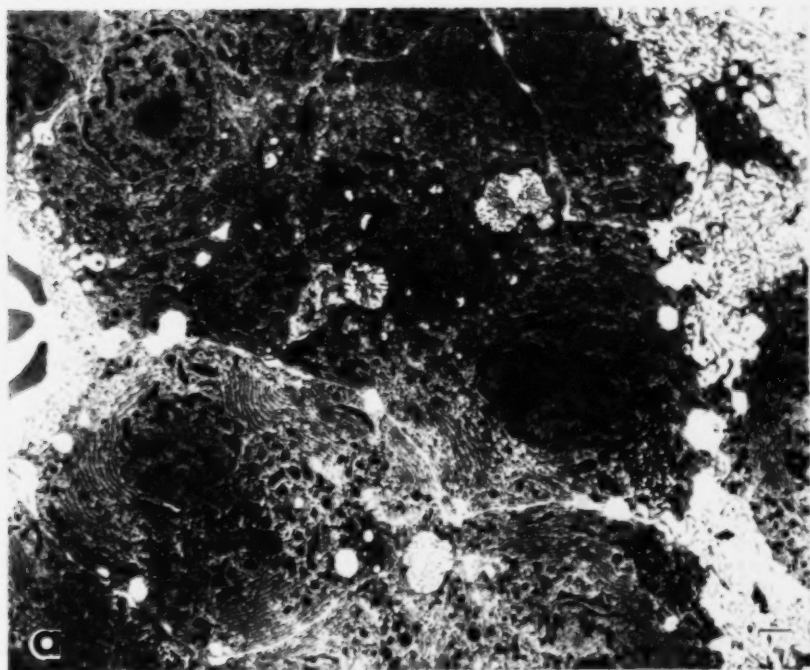
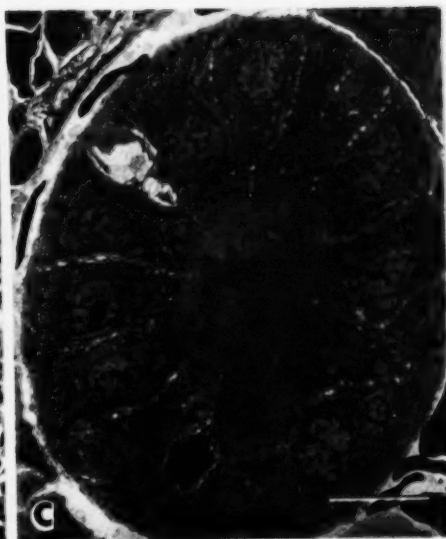
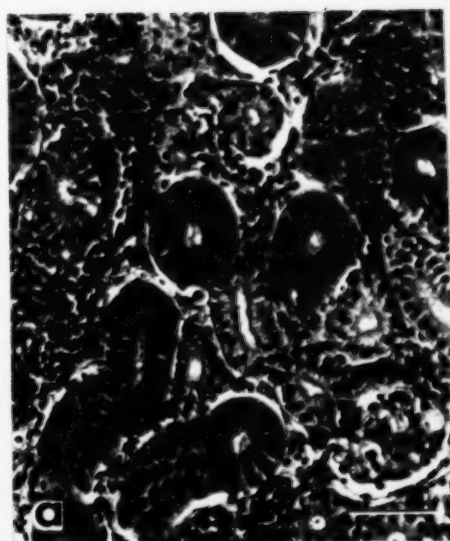


Figure 3. Light and electron micrographs of the posterior kidney from a control fish (a+c, respectively) and a fish in $1,000 \mu\text{g kg}^{-1}$ dose group which survived the 26 h (b+d, respectively). Note the occurrence of completely destroyed tubular epithelial cells adjacent to seemingly healthy tubules in b), and the dilation of Bowman's space. Also note the difference in size between the control (c) and treated (d) tubules, and the complete destruction of epithelial cells in the treated fish (d). All that remains of the treated tubule is an intact basement membrane. Bars in light micrographs are $50 \mu\text{m}$ and those in the electron micrographs are $10 \mu\text{m}$.



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Chapter 8

Oral and intraperitoneal toxicity of microcystin-LR to rainbow trout (*Oncorhynchus mykiss*)⁶

Introduction

Cyanobacteria (blue-green algae) commonly occur in nutrient-rich fresh waters worldwide and can cause substantive water quality problems including formation of aesthetically-unappealing green and blue-green colored scums along shorelines and the production of musty odors and off-tastes of potable water (Persson 1983; Kenefick et al. 1992; Hruddy et al. 1993). In addition, several species of cyanobacteria produce potent neuro- and hepatotoxic compounds (Carmichael 1992). Microcystin-LR (MC-LR) is one of more than 50 cyclic peptide hepatotoxins produced primarily by the cyanobacterium, *Microcystis aeruginosa* (Carmichael 1994; Rinehart et al. 1994) and is commonly detected in phytoplankton during the summer and autumn months in many lakes in central Alberta (Kotak et al. 1993b, 1995).

Accidental ingestion of microcystin-LR has caused poisonings and deaths of livestock, wildlife and family pets (Schwimmer and Schwimmer 1968) after the animals consume toxin-containing cells and/or water. Intraperitoneal injection of MC-LR or microcystin-containing extracts of cyanobacteria in laboratory mice can cause massive hemorrhage of the liver and death in less than 1 h (Kotak et al. 1993a+b). Microcystins have also been suspected as a contributing factor to massive die-offs of fish during the summer months (i.e., summerkill) in lakes containing high concentrations of cyanobacteria.

Although the intraperitoneal toxicity and organ pathology of microcystins in a few fish species has been demonstrated (e.g., Phillips et al. 1985; Sugaya et al. 1990; Rabergh et al. 1991; Andersen et al. 1993; Tencalla et al. 1994; Kotak et al., submitted a), few have

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examined natural routes of exposure (uptake of ingested microcystins and across the gill surface) in the laboratory or directly within lakes containing toxin-producing cyanobacteria. Rodger et al. (1994) reported liver and gill lesions in moribund brown trout (*Salmo trutta*) during an extensive bloom of the cyanobacterium, *Anabaena flos-aquae*, that was producing MC-LR and several other microcystins in Loch Leven, Scotland. Because the liver lesions were similar to those observed in fish injected intraperitoneally with MC-LR or microcystin-containing cyanobacterial extracts, Rodger et al. (1994) suggested that the die-off (approximately 1000 fish) may have been due to microcystin toxicity. However, a direct link between the toxic cyanobacterium and the fish (e.g., detection of MC-LR in the fish livers) was not made. Tencalla et al. (1994) found that MC-LR was lethal to rainbow trout by oral exposure at a dose of $6,600 \mu\text{g kg}^{-1}$ but did not provide micrographs of the organ pathology (e.g., histological and ultrastructural lesions) caused by the toxin. Thus the pathology of orally-administered MC-LR in fish is lacking.

The objectives of this study were to examine: 1) the comparative toxicity of intraperitoneally- and orally-administered MC-LR in rainbow trout, 2) the effects of MC-LR on the plasma activities of the enzymes lactate dehydrogenase, aspartic acid aminotransferase and alanine aminotransferase (an indication of hepatic trauma), and 3) the organ pathology of MC-LR at the histological (i.e., light microscopy) and ultrastructural level of resolution.

Materials and Methods

Fish - Rainbow trout (*Oncorhynchus mykiss*) were obtained from Sun Valley Trout Farm, Mission, British Columbia and weighed 118 ± 5 ($\pm$ SE) and 192 ± 8 g for the intraperitoneal (i.p.) and oral trials respectively. Fish were held in circular 680 L tanks with 15°C dechlorinated City of Edmonton water, recirculated at 3 L min^{-1} . Dissolved oxygen was maintained at 9.1 ± 0.1 (SE) mg L^{-1} . Fish were fed commercial trout chow (Martin Feed Mills, Elmira and Tavistock, Ontario) at 3% of body weight d^{-1} . Fish were maintained according to standard husbandry practices (Canadian Council of Animal Care

1984) and were allowed to acclimate for 14 d prior to the trials. Food was withheld from the fish 48 h prior to experimentation.

In the i.p. toxicity trial, fish were weighed in a tared vessel containing water and randomly assigned to one of seven treatment groups: negative control (no injection), saline control (saline injection), and MC-LR (>95% pure and obtained from Calbiochem, U.S.A.) doses of 50, 100, 200, 400 and 800 $\mu\text{g kg}^{-1}$ in saline, with five fish per treatment. Intraperitoneal doses were injected along the ventral midline anterior to the pelvic fin with 1 cm, 22 gauge needles. Volumes injected ranged from 0.1 to 0.3 mL.

For the oral toxicity trial fish were weighed and MC-LR doses constructed by the following method: MC-LR was dissolved in methanol and an appropriate volume (based on the mass of the fish and the concentration of MC-LR in the stock methanol solution) transferred to gelatin capsules. The methanol was then evaporated overnight at room temperature. Methanol did not alter the integrity of the capsule. Each fish was then marked by clipping the tip of one fin (upper or lower caudal, anal, adipose, pelvic or pectoral) so that individual fish could be easily observed and identified. Fish were fin-clipped 72 h prior to gavaging. Fish were assigned to one of eight treatment groups: negative control (no fin clip, no capsule), fin clip control (no capsule), capsule control (saline), and MC-LR doses of 500, 1,000, 2,000, 4,000 and 8,000 $\mu\text{g kg}^{-1}$. To administer the capsule, each fish was restrained in a wet mesh net and the capsule was placed at the back of the mouth and gently pushed towards the esophagus. A natural reflex reaction caused the capsule to be swallowed. In both trials, fish were observed continuously for the first 12 h and then every 3-5 h for the duration of the trials (96 h). Capsules were never regurgitated, which would have been easily noticed as the capsules tended to float in water. To ensure that the capsules were digested, an additional four fish each received an empty capsule and then were sacrificed after 4, 8, 18 and 24 h. The capsules were completely dissolved within 8 h.

Moribund fish and those surviving to the termination of the experiments (96 h) were sacrificed by a sharp blow to the head. Fish were weighed and blood collected from the caudal vein in a vacuutainer containing EDTA. Blood was immediately placed on ice in preparation for enzyme analyses. A complete necropsy was then performed and gross

lesions of the internal organs were recorded. The livers were excised, freed of attached tissue and then weighed, with the exception of the negative control group in the oral trial. Livers in this treatment were not weighed. The mass of the livers were then expressed as a percentage of the total fish mass.

Biochemical analyses of plasma - Red cells were removed from plasma by centrifugation (3,000g for 10 min). Plasma was analyzed for the enzymes lactate dehydrogenase (LDH), aspartic acid aminotransferase (AST) and alanine aminotransferase (ALT) on a Hatachi Autoanalyzer, at 25 °C. Activities are reported as U L⁻¹, which is defined as the amount of enzyme that will convert 1 µmol of substrate per min under specified conditions in the assay.

Histopathology and ultrastructure - For both toxicity trials, sections (5 mm) of liver and posterior kidney were fixed in Bouin's fixative, processed and embedded in paraffin, cut at 4-6 µm thickness, stained with H&E and examined by light microscopy. In addition, sections of pyloric caecae and intestine were processed as above for the oral trial. Liver lesions were scored on the basis of their distribution within the lobule (periportal, midzonal or centrilobular) and the entire liver (focal, multifocal or massive). In addition, the percentage of the liver affected was estimated. For all other organs examined, the distribution of the lesions in the entire organ and the % of the organ affected was determined. Thin sections (0.5 to 1.0 mm) of liver and kidney were processed routinely for TEM (Kotak et al. submitted a). Ultrastructural lesions were examined on a Hatachi H-6000 transmission electron microscope.

Statistical Analyses - Mean percent of liver to body mass ratios and plasma enzyme activities (U L⁻¹) were computed for each treatment group and each MC-LR treatment was then compared to controls groups by *t*-test. A significant difference was defined as *P*<0.05. Since livers from fish in the negative control group of the oral toxicity trial were not weighed, and thus the percent liver to body mass ratio could not be computed, ratios from fish of the corresponding control group in the i.p. trial (i.e., negative control) were used in the oral trial *t*-test. With two exceptions, no significant differences were found between the various control groups (e.g., between the negative control and saline control groups in the i.p. trial by *t*-test, and between the negative

control, fin clip control and saline capsule control groups in the oral trial by one-way ANOVA) with respect to percent liver to body mass ratio and plasma enzymes (data not shown). Therefore, data for all control groups within each trial were combined prior to comparisons with MC-LR dose groups. However, because fish in the saline control group had significantly higher AST and ALT activities than negative control fish in the i.p. trial, all MC-LR dose groups for these two enzymes were compared with saline control fish only. Thus these two analyses were conservative. Student's *t*-tests and one-way ANOVAs were performed on SPSS for Windows 6.0 (SPSS Inc., Chicago, IL.).

Results

Behavior and mortality - In the intraperitoneal (i.p.) trial, fish injected with saline or microcystin-LR (MC-LR) at a dose of 50 or 100 $\mu\text{g kg}^{-1}$ were initially lethargic immediately after injection but resumed normal swimming activity within 1 to 2 h. In contrast, fish injected with a dose of 200 $\mu\text{g kg}^{-1}$ MC-LR or greater remained sluggish until death or termination of the trial. Fish in the 400 and 800 $\mu\text{g kg}^{-1}$ treatments in particular displayed severely altered behavior. Extended periods of lethargy were interrupted by sudden episodes of frenetic swimming. Generally, fish in the two highest doses had difficulty in maintaining buoyancy control such that they commonly floated on their sides near the surface of the water or with their head facing vertically down. In these fish, ventilatory movements of the operculum gradually decreased in frequency and magnitude over the trial. While fish in the saline, 50 and 100 $\mu\text{g kg}^{-1}$ treatments were light silvery-green in color, the dorsum of fish in the higher dose treatments gradually darkened to an olive-green. One of five fish in the 200 $\mu\text{g kg}^{-1}$ treatment died (at 37 h post injection [p.i.]), three of five fish in the 400 $\mu\text{g kg}^{-1}$ treatment (at 29, 30 and 46 hours p.i.) and three of five fish in the 800 $\mu\text{g kg}^{-1}$ treatment (at 11, 26 and 39 hours p.i.). No mortality occurred in the negative or saline controls or in the 50 and 100 $\mu\text{g kg}^{-1}$ MC-LR treatments.

In the oral trial, fin clipped fish displayed no signs of stress 72 h after being marked. Fish receiving gelatin capsules resumed normal swimming activity 2 to 4 h after

gavaging. Only fish in the 8,000 $\mu\text{g kg}^{-1}$ treatment displayed a color change and lethargic behavior that extended throughout the length of the trial. As with fish in the higher dose treatments in the i.p. trial, these fish had difficulty maintaining buoyancy control. One of five fish died in each of the 1,000, 2,000, 4,000 and 8,000 $\mu\text{g kg}^{-1}$ treatments, at 26, 78, 6 and 28 hours p.i., respectively. The moribund fish in the 4,000 $\mu\text{g kg}^{-1}$ treatment likely died from handling stress.

Gross pathology - With the exception of the 100 $\mu\text{g kg}^{-1}$ treatment, the percent liver to body mass ratio in fish from MC-LR treatments in the i.p. trial were higher than control fish ($P < 0.01$; Fig. 1a). While percent liver to body mass ratios for control fish were 0.84 to 0.89 on average, ratios were up to 1.4-fold higher on average in MC-LR treatments. In the oral trial, except the lowest dose group (500 $\mu\text{g kg}^{-1}$), the percent liver to body mass ratio in fish in all MC-LR treatments were significantly higher than control fish ($P < 0.005$; Fig. 2a). The highest ratio on average (1.21) was from fish in the 2,000 $\mu\text{g kg}^{-1}$ treatment.

The liver was the only organ with gross pathological changes. In both trials, livers from control fish were firm and dark red. In contrast, gross changes in the liver were evident in fish from the lowest MC-LR treatments in the i.p and oral trial (50 and 500 $\mu\text{g kg}^{-1}$, respectively). Livers in these MC-LR-treated fish were red with alternating pale tan-colored patches, with occasional small foci of hemorrhage. The livers in fish from both trials progressively changed from being firm and red to wet, soft and pale tan in color throughout the entire liver with increasing MC-LR dose. In the i.p. trial, livers further progressed to a cream color in fish injected with 200 $\mu\text{g kg}^{-1}$ MC-LR or more. Livers of fish in the oral trial did not progress in color beyond pale tan. In contrast to livers from control fish which oozed blood when sectioned, straw-colored fluid oozed from the pale tan and cream colored livers of MC-LR-treated fish. A straw-colored fluid was found in the coelomic cavity of all fish treated with MC-LR.

Jaundice or yellowing of the membranous parts around the eyes, operculum and fins, and the belly was evident in two fish in each of the 400 and 800 $\mu\text{g kg}^{-1}$ treatments in the i.p. trial. One fish was jaundiced in the 8,000 $\mu\text{g kg}^{-1}$ treatment in the oral trial. Bile

color changed from dark green to a light yellow-green in all fish given a dose of 100 $\mu\text{g kg}^{-1}$ or greater and in one of five fish in the 50 $\mu\text{g kg}^{-1}$ treatment in the i.p. trial. A similar color change of the bile was evident in fish given a dose of 2,000 $\mu\text{g kg}^{-1}$ MC-LR or greater in the oral trial.

Plasma enzymes - Plasma activities of LDH, AST and ALT in fish from MC-LR treatments in the i.p. trial generally increased with increasing MC-LR dose up to a dose of 400 $\mu\text{g kg}^{-1}$, and then decreased slightly in the 800 $\mu\text{g kg}^{-1}$ treatment (Fig. 1b,c +d). Average LDH, AST and ALT activities in the 400 $\mu\text{g kg}^{-1}$ treatment peaked at 9995, 3104 and 795 U L^{-1} . Plasma enzyme activities were significantly higher in the MC-LR treatments than in control fish for all three enzymes with one exception. AST activity in the 50 $\mu\text{g kg}^{-1}$ treatment was not significantly different from control fish (Fig. 1c). Patterns of enzyme activities in fish from the oral trial were less consistent than in the i.p. trial. For example, AST and ALT activities in the 2,000 and 4,000 $\mu\text{g kg}^{-1}$ treatments were lower than in the 1,000 $\mu\text{g kg}^{-1}$ treatment (Fig. 2c+d). However, enzyme activities in most MC-LR treatments were significantly higher than in control fish (Fig. 2b, c+d). Plasma activities of LDH, AST and ALT in the 500 $\mu\text{g kg}^{-1}$ treatment in the oral trial were 11.1, 7.9 and 17.3-fold, respectively, lower than activities in a roughly equivalent dose (400 $\mu\text{g kg}^{-1}$) in the i.p. trial. Similarly, plasma activities of LDH, AST and ALT in the 1,000 $\mu\text{g kg}^{-1}$ treatment in the oral trial were 6.1, 3.1 and 6.9-fold, respectively, lower than activities in a similar dose (800 $\mu\text{g kg}^{-1}$) in the i.p. trial.

Histopathology and ultrastructure of organs - Pathological changes to the liver of MC-LR-treated fish in both trials increased with increasing dose and was most severe in fish that survived the 96 h trials. Liver lesions from MC-LR-treated fish in both trials were similar, although the severity and extent of damage was much less in the oral trial. Histologically, the mildest liver lesions observed in both trials consisted of hepatocellular swelling (hydropic degeneration), causing obliteration of sinusoids, and dissociation of hepatocytes. In the oral trial these lesions occurred as a single focus in fish of the 500 $\mu\text{g kg}^{-1}$ treatment (Fig. 3b) and as multifocal lesions at higher doses, and in the i.p. trial as multifocal lesions in fish of the 50 $\mu\text{g kg}^{-1}$ treatment and involved the entire liver (i.e., the

lesion was diffuse) at higher doses (Tables 1 and 2). While less than 30% of the liver had lesions in the oral trial, an average of 70% of the liver had lesions in the i.p. trial (Tables 1 and 2). The cytoplasm of hepatocytes from fish treated with a dose of 1,000 $\mu\text{g kg}^{-1}$ or greater in the oral trial and all MC-LR-treated fish in the i.p. trial were also highly vacuolated and contained pyknotic nuclei (Fig. 3c). Hepatocyte necrosis was evident in the livers of fish even at the lowest MC-LR doses in both trials but the necrotic lesions were more pervasive in the fish from the i.p. trial (Tables 1 and 2). At the lower doses in both trials, lesions consisted of variably-sized foci of coagulative necrosis (Fig. 3c) commonly surrounding the central veins. In fish in the higher MC-LR treatments ($>2,000$ and $>100 \mu\text{g kg}^{-1}$ MC-LR in the oral and i.p. trials, respectively), hepatocyte necrosis was liquifactive rather than coagulative. This lesion was characterized by hepatocyte cell membranes which lysed, leaving a milieu of cellular debris floating in a proteinaceous matrix (Fig. 3d). In these areas, the only residual cells were often short chords of regenerative endplate hepatocytes or proliferative bile ducts. In the i.p. trial, coagulative and liquifactive necrosis was also observed subjacent to Glisson's capsule, evidence for transport of MC-LR across the liver capsule. Occasionally, sloughed hepatocytes were found in the central veins in MC-LR-treated fish, which presumably might travel to, and become trapped in, the fine capillaries of the gills, although hepatocytes were never detected in the gill capillaries. Hemorrhage was absent or rare in the livers from MC-LR-treated fish. Hepatocytes from control fish were irregular in shape, contained large nuclei and chords of hepatocytes were separated by well-defined sinusoids (Fig. 3a), and therefore displayed none of the pathological lesions described in the MC-LR-treated fish above.

Ultrastructural lesions in the liver were identical in MC-LR-treated fish from both trials, although, as was the case for lesions observed by light microscopy, the lesions were more severe and pervasive in fish from the i.p. trial. In both trials, ultrastructural changes in the liver from fish that were treated with MC-LR appeared to progress in severity from generalized swelling of the cells and organelles (e.g., mitochondria), to loss of ribosomes from and subsequent dilation (vesiculation) of the endoplasmic reticulum. In addition, numerous electronlucent, membrane-bound vacuoles were evident in necrotic hepatocytes.

In zones of liquifactive necrosis, ruptured plasma membranes resulted in a matrix of cellular debris (Fig. 4b) in which organelles were usually no longer discernible. In contrast to fish treated with MC-LR, hepatocytes from all control fish in both trials were irregular in shape, had a well-defined plasma membranes and endoplasmic reticulum with attached ribosomes and lacked electronlucent vacuoles (Fig. 4a).

Histological lesions in the kidney were observed only in fish from the highest MC-LR treatment groups in both trials. Lesions ranged from swelling (enlargement) of the tubules and a dilation of Bowman's space (Fig. 5b), to coagulative and liquifactive necrosis of the tubule epithelial cells. In tubules from fish of the $8,000 \mu\text{g kg}^{-1}$ treatment in the oral trial, some of the cell membranes lysed resulting in an isolated milieu of cellular debris, as was the case in the liver. In fish in the $800 \mu\text{g kg}^{-1}$ treatment in the i.p. trial, tubular necrosis was more extensive than in the oral trial, with liquifactive necrosis involving the entire tubules. These necrotic tubules were commonly surrounded by seemingly normal tubules. No changes were detected in the kidney from any of the control fish in both trials (Fig. 5a).

The prominent ultrastructural lesion in the kidney from the fish displaying histological lesions was tubular cell necrosis (Fig. 6b). The formation of this lesion appeared to progress from tubular cell and mitochondrial swelling and loss of ribosomes from endoplasmic reticulum, to the vesiculation of endoplasmic reticulum (Fig. 6b, inset). In the most severe case (in fish from the i.p. trial), liquifactive necrosis involved entire tubules such that organelles floating in a milieu of cellular debris, similar to that of the hepatocyte liquifactive necrosis described previously. In these cases, only the basal lamina of the tubule remained. As with the most severe liver lesions, dense, membrane-bound vacuoles were also present in the kidney (not shown). No such lesions were found in the kidney of any of the control fish from both trials (Fig. 6a).

Histological lesions of the pyloric caecae and intestine were also present in fish exposed to MC-LR in the oral study. Lesions consisted of focal to multifocal necrosis and lysis of epithelial cells making up the villi (Fig. 7) and were evident in fish from all MC-LR treatment groups but not in control fish.

Discussion

Microcystin-LR (MC-LR) caused severe liver damage in the fish exposed by intraperitoneal (i.p.) injection and oral gavage, and at higher doses, even death. The hallmark or characteristic lesion caused by MC-LR in the fish was liquifactive necrosis, in which rupture of the plasma membrane resulted in a milieu of cellular debris (Fig. 3d, 4b). At the ultrastructural level of resolution, the lesion appeared to progress from hepatocyte swelling with the obliteration of sinusoids, followed by hepatocyte dissociation, swelling of mitochondria and loss of ribosomes from endoplasmic reticulum (ER) and vesiculation of the ER causing vacuolization of the cytoplasm in the hepatocytes. This was followed by nuclear pyknosis and ultimately the lysis of the plasma membrane (Fig. 4b). At this final stage, organelles were no longer discernible. Most of the described pathological changes in the fish in the present study at the light and electron microscopy level of resolution are consistent with those reported in carp (*Cyprinus carpio*) by Rabergh et al. (1991) and rainbow trout by Phillips et al. (1985) and Tencalla et al. (1994). However, liquifactive hepatocyte necrosis was only reported in the study by Tencalla et al. (1994), although they did not provide micrographs to support their pathological descriptions. I have previously observed this type of lesion in rainbow trout exposed i.p. to MC-LR (Kotak et al. submitted a).

In contrast to the primary liver lesion in fish, mammals exposed to microcystins display a liver lesion that is characterized by massive hemorrhage (Falconer et al. 1981; Kotak et al. submitted a) in which the liver becomes greatly engorged with blood. While upon sectioning, blood oozes out of a liver from a mammal exposed to microcystins, straw-colored fluid oozed out the livers of fish exposed to MC-LR. The increase in liver mass, observed in both mammals (Kotak et al. 1993a+b, submitted a) and fish in the present study are therefore likely caused by different mechanisms (engorgement by blood influx in mammals, and retention of water by the liver in fish). Hemorrhage was virtually absent in fish that were exposed to MC-LR in the present study. It therefore seems likely that the ultimate cause of death would also differ. Mice exposed i.p. to MC-LR die of hemorrhagic shock (Kotak et al. 1993b, submitted a) whereas the fish likely die of general hepatic failure. Despite these marked differences, the two groups of animals do share

some common lesions in the liver including cell swelling, nuclear pyknosis, swelling of mitochondria, and vesiculation and loss of ribosomes (degranulation) of the ER (Dabholkar and Carmichael 1987; Berg et al. 1988; Hooser et al. 1990). Interestingly however, Sugaya et al. (1990) reported hemorrhage in the liver of goldfish (*Carassius auratus*) exposed i.p. to another microcystin analogue, MC-RR, although they present no histological or ultrastructural evidence. The reason for the difference in liver pathology between my studies on rainbow trout and those of Rabergh et al. (1991), with Sugaya et al. (1990) is unclear.

While MC-LR is classified as a hepatotoxin, there is also evidence showing that the toxin has effects on other cell types. I found that i.p. and orally-administered MC-LR at the two highest doses caused dilation of Bowman's space (Fig. 5b) and coagulative to liquifactive necrosis of tubular epithelial cells of the kidney (Fig. 6b). In the most severe cases (e.g., in fish from the highest i.p. treatment) all epithelial cells in the tubule were destroyed, leaving only the basal lamina of the tubule. Such destruction of the kidney, an organ responsible in part for water balance, may explain the observed water retention in the liver. Exposure to MC-LR also caused a dilation of Bowman's space, likely caused by a backup of filtrate within Bowman's capsule. I have previously reported such kidney lesions in rainbow trout exposed to an i.p. dose of $1,000 \mu\text{g kg}^{-1}$ MC-LR (Kotak et al. submitted a). Rabergh et al. (1991) also noted necrosis of epithelial cells in the kidney tubules of carp exposed i.p. to MC-LR, although the lesions were not as severe as in my study, probably due to the shorter duration of their trials (24 h).

These liver lesions reported in the present study are consistent with those that I reported previously (Kotak et al. submitted a) in rainbow trout exposed i.p. to doses of 400 and $1,000 \mu\text{g kg}^{-1}$ MC-LR. However, lesions observed in the livers of fish from the 50, 100 and $200 \mu\text{g kg}^{-1}$ treatments in the present study were more severe and pervasive than those in the $400 \mu\text{g kg}^{-1}$ treatment reported by Kotak et al. (submitted a). This may be due to the duration of each experiment. While the present study lasted 96 h, my previous study (Kotak et al. submitted a) was only 26 h. Therefore, the differences may be ascribed to exposure time. This may also be evident in differences between the two studies with respect to mortality. While Kotak et al. (submitted a) reported 100%

mortality in the 1,000 but no mortality in the 400 $\mu\text{g kg}^{-1}$ treatment, there was 20, 60 and 60% mortality in the 200, 400 and 800 $\mu\text{g kg}^{-1}$ treatments, respectively, in the present study. These doses are considerably higher than the reported i.p. LD_{50} for mice (50 $\mu\text{g kg}^{-1}$, Carmichael 1988). Combining the data from my present and previous studies would give an approximate LD_{50} of 500 $\mu\text{g kg}^{-1}$, ten times higher than that for mice. The fish in this study also survived a lethal dose of MC-LR much longer than do mice. The mean survival time in the i.p. trial in the present study was 31 h. In contrast the mean survival time in mice is 1-2 h (Kotak et al. 1993a+b, submitted a).

Two other studies have evaluated the i.p. toxicity of MC-LR in rainbow trout. Tencalla et al. (1994) reported 100% mortality (2 fish) within 24 h in trout that were exposed i.p. to either purified MC-LR (at 550 $\mu\text{g kg}^{-1}$) or a *Microcystis aeruginosa* extract containing MC-LR at the same dose. Phillips et al. (1985) also reported mortality within 36 h of trout injected with a *M. aeruginosa* extract equivalent to the i.p. LD_{50} in mice, although the number of fish mortalities was not reported. One hundred percent mortality was not observed in my study at i.p. doses up to 800 $\mu\text{g kg}^{-1}$. The higher toxicity reported in these studies relative to the present study, may be due to the smaller fish used in their experiments (30-60 g versus 118 g in the present study). The older fish used in my trials may possess a more complete or sophisticated system for detoxifying such toxins, and thus would be more physiologically resistant to the toxin.

There also appears to be some variation in the dose tolerance of different fish species to microcystins. Andersen et al. (1993) injected Atlantic salmon (*Salmo salar*) with 3 successive doses (spaced 3 d apart) of MC-LR at 555 $\mu\text{g kg}^{-1}$ per injection. Only two fish died or were moribund over the 36-d experiment. In an experiment examining the toxicity of another microcystin analogue, MC-RR, to goldfish (*Carassius auratus*) Sugaya et al. (1990) reported an i.p. LD_{50} of over 2,000 $\mu\text{g kg}^{-1}$. However, part of the higher dose tolerance can be explained by lower toxicity of MC-RR. The LD_{50} of MC-RR in mice can be 5 to 16 times higher than that of MC-LR (see Table 2, Chapter 2). In contrast, Rabergh et al. (1991) found that the i.p. LD_{50} of MC-LR to carp (*Cyprinus*

carpio) was between 300 and 550 $\mu\text{g kg}^{-1}$, and in a second trial was 80 $\mu\text{g kg}^{-1}$. Thus, there appears to be variation in the susceptibility of different fish species to microcystins.

In the oral exposure trial, I observed mild liver damage in rainbow trout exposed to a dose of 500 $\mu\text{g kg}^{-1}$ MC-LR (the lowest dose studied) and massive destruction of the liver and kidney at the highest dose (8,000 $\mu\text{g kg}^{-1}$). Despite the much lower doses in the i.p. compared to the oral trial, mortality was much higher and liver damage was more severe (as assessed by light microscopy and plasma enzyme activities) in the i.p. trial. This is not surprising since i.p. exposure is a rapid and direct route of exposure. A high percentage of the administered dose would be expected to reach the liver. In contrast, for MC-LR to reach the liver after oral gavage, the toxin must cross a number of biological barriers, including the intestinal wall. Since the extent of liver lesions by light microscopy (Tables 1 and 2) in the oral trial was 7 to 8-fold lower than roughly similar doses (on a mass basis) in the i.p. trial, much of the orally-administered toxin may have not crossed the intestinal wall, and therefore little entered the bloodstream to be delivered to the liver. This toxin would end up being excreted in the feces. Alternatively, since intestinal epithelial cells in some animals (e.g., chicken) have been shown to take up microcystins (and be destroyed, Falconer et al. 1992), some of the orally-administered MC-LR in the fish may have been tied up in the intestinal cells. Uptake of microcystins is highly organ-specific and occurs in cell types containing multispecific bile acid or similar transporters, such as hepatocytes, enterocytes of the intestine and in kidney tubular cells (Runnegar et al. 1981; Eriksson et al. 1987, 1990; Dahlem et al. 1989; Rabergh et al. 1991; Kotak et al. submitted a). In the present study I observed destruction of cells making up the villi of the intestine and pyloric caecae, suggesting a similar transport mechanism may be operating in these tissues.

Few studies have examined the oral toxicity of microcystins to fish. Tencalla et al. (1994) found that while oral doses of purified MC-LR at 1,200 $\mu\text{g kg}^{-1}$ or *M. aeruginosa* extracts equivalent to 1,700 $\mu\text{g kg}^{-1}$ MC-LR did not cause histological lesions in the liver or mortality of rainbow trout within 96 h, repeated doses (8 times at 12 h intervals at 550 $\mu\text{g kg}^{-1}$ gavage⁻¹) caused slight liver damage. Additionally, a single oral dose of 6,600 $\mu\text{g kg}^{-1}$ MC-LR was lethal within 72 to 96 h. Gross pathology (pale colored livers) and

descriptions of histological lesions in Tencalla's study, including liquifactive hepatocyte necrosis, are consistent with my results.

In both i.p. and oral trials I used the activities of the enzymes LDH, AST and ALT in the plasma as an index of hepatic trauma. It is evident from the differences in magnitude of enzyme activities that liver damage was much more extensive and severe in the i.p. versus the oral trial (Figs. 1+2). My results are consistent with those presented by Rabergh et al. (1991) and Tencalla et al. (1994) examining the toxicity of i.p. and orally-administered MC-LR, respectively, in carp and rainbow trout, respectively. While enzyme activities in my study were higher in MC-LR-treated fish than in control fish, the results were not always consistent. For example, enzyme activities did not always increase with increasing dose (see Fig. 2b,c+d) and in one case in a high dose treatment ($4,000 \mu\text{g kg}^{-1}$, oral trial) ALT activity was not significantly different from control fish (Fig. 2d), despite severe liver damage observed in histological and ultrastructural examinations. Therefore, although quicker, cheaper and easier to perform than light and electron microscopic examinations of tissues to define organ damage, caution should be exercised when interpreting such enzyme data on their own. When used in conjunction with histological or ultrastructural evidence, plasma enzyme assessments can be highly useful.

Given that microcystins are toxic to rainbow trout by an oral route of exposure, the questions remain as to whether fish are exposed to such concentrations in the natural environment, and whether such exposure may cause pathological effects, or in the worst case, mortality of fish. It is possible that certain fish species could potentially be exposed to microcystins continuously over open water seasons in lakes through their food base. For example, accumulation of MC-LR has been documented in natural communities of zooplankton (containing cladocerans and copepods) and in three genera of gastropods (Kotak et al. submitted b) at concentrations up to 67 and $120 \mu\text{g g}^{-1}$ (dry weight), respectively in the two invertebrate groups in two hypereutrophic lakes in central Alberta. An assessment of exposure for a fish might be the following: a 100 g fish that consumes 3% of its body weight per day of gastropod tissue would consume 12 g (wet weight) of gastropod tissue in 96 h (the length of my oral trial). If the gastropods contained $120 \mu\text{g g}^{-1}$ dry weight or $12 \mu\text{g g}^{-1}$ wet weight of MC-LR, then the fish would consume $144 \mu\text{g}$ of

MC-LR in the four days of feeding. Since I observed pathological changes in the livers of rainbow trout exposed orally to a dose of 500 ug kg⁻¹ (a dose of 50 ug for a 100 g fish) is conceivable that, particularly if the hypothetical fish feeds on microcystin-containing gastropods all summer, the fish could develop severe hepatocyte necrosis. With continual exposure, death may also result. Tencalla et al. (1994) performed a similar risk assessment and concluded that it is highly likely that fish species that feed directly on toxic phytoplankton could develop severe liver lesions, which may lead to death. Fish mortality caused by ingestion of microcystin-containing food stuffs may offer an alternative hypothesis to explaining the sporadic, summerkill events in hypereutrophic lakes when dissolved oxygen (the lack of which is usually pointed out as the factor leading to such fish die-offs) was sufficient to support fish.

A series of studies have been published that lend credence to the hypothesis that microcystins present in ingested food stuffs can cause spectacularly large, and economically devastating fish kills. For example, rearing of Atlantic salmon along the west coasts of British Columbia and the State of Washington is a multi-million dollar industry. Kent et al. (1988) and Kent (1990) reported substantive mortalities in net pen-reared salmon since 1986 and that all affected fish displayed severe liver lesions which they termed netpen liver disease (NLD). NLD in the affected fish was not related to commercial fish food, could not be experimentally transmitted (therefore was not of bacterial or viral origin), was not related to anthropogenic contamination and progressed in severity over the summer in each year. They thus suspected a natural toxin. Because of the similarity to the liver pathology observed in published studies with laboratory fish exposed i.p. to microcystins, Andersen et al. (1993) examined the pathology of i.p.-administered MC-LR in healthy salmon. They successfully reproduced the NLD lesions in the i.p.-treated fish. While diffuse hepatocyte necrosis was the main lesion observed in the liver of salmon, as with my and other studies involving exposure of fish to microcystins, Andersen et al. (1993) also reported hepatic megalocytosis, a lesion not reported in any other fish or mammal exposed to microcystins. Kent et al. (1988) and Kent (1990) also observed "islands" of regenerative hepatocytes in salmon afflicted with NLD and in those that were transferred to clean sea water. I also observed zones of regenerative endplate

hepatocytes scattered amongst massive zones of necrotic hepatocytes in both my i.p. and oral studies. Lastly, further work provided strong evidence to suggest that MC-LR, produced in the marine environment, was responsible for NLD. Andersen et al. (1993) successfully isolated a compound from the livers of fish afflicted with NLD and from biota fouling the net pens which was chromatographically-indistinguishable from MC-LR. MC-LR was not detected in the livers of salmon not afflicted with NLD. They surmised that the salmon were exposed to the toxin by feeding on the microcystin-containing biota on the net pens. Therefore, it is likely that the health of fish which either feed directly on toxic phytoplankton or food stuffs that have accumulated the toxin could be compromised, even to the point of death.

One other natural route of exposure of fish to microcystins may also be possible: uptake of the dissolved toxin across the gills. This route of exposure is commonly cited as a contributing factor or the main cause of fish kills in hypereutrophic lakes when dissolved oxygen data from the water body in question indicate that oxygen concentrations were well above those required for fish to survive. However, there is little concrete field data to support this hypothesis. Such a mortality event would be assumed to occur when an algal (cyanobacterial) bloom senesces, releasing the intracellular microcystins into the water column, with subsequent uptake of the toxin across the gills. Gaete et al. (1994) found that microcystins in an extract of hepatotoxic *M. aeruginosa* inhibited ion pump enzymes in microsomal fractions of gills isolated from carp, probably through the inhibition of protein phosphatases. They suggest that uptake of dissolved microcystins may lead to loss of ion homeostasis and possibly death in fish. In partial support of this hypothesis, Rodger et al. (1994) reported pathological lesions in the gill and liver of moribund brown trout (*Salmo trutta*) collected from Loch Leven, Scotland during an extensive bloom of the cyanobacterium, *Anabaena flos-aquae*, that was producing several microcystin analogues. Approximately 1,000 fish died within a 24 h period. Gill pathology was indicative of acute irritation and the pathological lesions in the liver were consistent with exposure to a microcystin. Unfortunately, without additional supporting evidence (e.g., the detection of microcystins in the liver of affected fish) it is difficult to draw a direct link between the toxin-producing cyanobacterium and the fish kill event.

Conclusions

This is the first study to describe the pathological lesions in the liver, kidney and intestinal tract in rainbow trout exposed orally to microcystin-LR (MC-LR) as well as by intraperitoneal injection, a standard route of exposure for toxicity evaluation. Both intraperitoneal and oral exposure of rainbow trout to MC-LR caused mild to severe coagulative and liquifactive hepatocyte necrosis which differed substantively from lesions described in mammals. The extent of liver damage caused by oral exposure was about 10-20% and 6-33% that of equivalent i.p. doses based on histological examination and plasma enzymes, respectively, indicating that uptake of MC-LR from the digestive tract is not 100% efficient. In addition to liver damage, MC-LR caused necrosis of epithelial cells of the tubules of the kidney and a dilation of Bowman's space at the highest dose in the i.p. and oral trials, and destruction of the villi of the intestine and pyloric caecae in the oral trial. Up to 60% mortality occurred at the two highest doses in the i.p. trial while mortality never exceeded 20% in the oral trial, despite much higher dose regimes. These results indicate that ingestion of microcystin-containing food items by fish in lakes containing toxic cyanobacteria could affect the health of fish by damaging the liver, kidney and intestinal lining, and could potentially cause mortality. More attention needs to be directed towards establishing the link between toxin-producing cyanobacteria and fish health.

Table 1. Distribution and estimated percent of liver displaying mild and severe liver lesions in rainbow trout from each dose treatment from the intraperitoneal exposure trial.

Treatment ^{*1}	Hepatocellular swelling/obliteration of sinusoids		Coagulative and/or liquifactive hepatocyte necrosis	
	Distribution	% of liver affected	Distribution	% of liver affected
Controls	None		None	
50	Multifocal	35	Multifocal	50
100	Diffuse	70	Diffuse	80
200	Diffuse	70	Diffuse	80
400	Diffuse	70	Diffuse	80
800	Diffuse	70	Diffuse	70

^{*1} Microcystin-LR doses expressed as $\mu\text{g kg}^{-1}$

Table 2. Distribution and estimated percent of liver displaying mild and severe liver lesions in rainbow trout from each dose treatment from the oral exposure trial.

Treatment ^{*1}	Hepatocellular swelling/obliteration of sinusoids		Coagulative and/or liquifactive hepatocyte necrosis	
	Distribution	% of liver affected	Distribution	% of liver affected
Controls	None		None	
500	Focal	10	Focal	10
1,000	Multifocal	10	Multifocal	10
2,000	Multifocal	10	Multifocal	20
4,000	Multifocal	10	Multifocal	10
8,000	Multifocal	30	Multifocal	50

^{*1} Microcystin-LR doses expressed as $\mu\text{g kg}^{-1}$

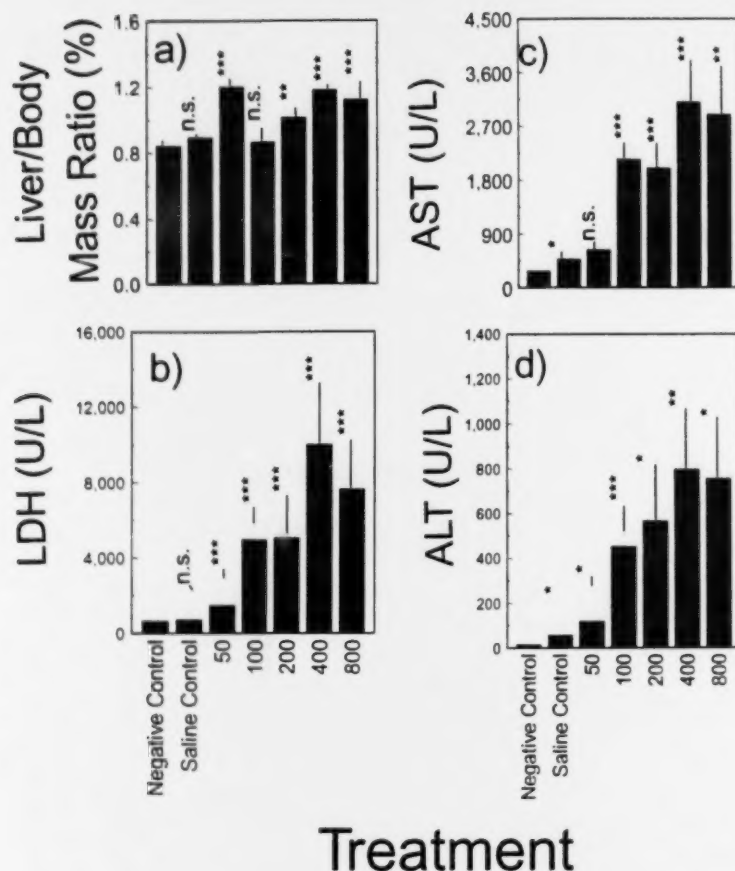


Figure 1. Average a) percent ratio of liver to body mass, and plasma activities (U L^{-1}) of b) lactate dehydrogenase, c) aspartic acid aminotransferase, and d) alanine aminotransferase in rainbow trout exposed intraperitoneally to microcystin-LR. Fish in the negative control group were not injected, in the saline control treatment were injected with physiological saline and microcystin-LR doses are reported as $\mu\text{g kg}^{-1}$. Vertical bars are standard error of the treatment mean. Levels of significance: n.s. = not significant ($P > 0.05$), * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.005$. The saline control treatment was compared to the negative control by *t*-test and then each microcystin-LR treatment in a) and b) was compared to the negative and saline controls combined by *t*-test while each microcystin-LR treatment in c) and d) was compared to the saline treatment only by *t*-test (see Methods and Materials section).

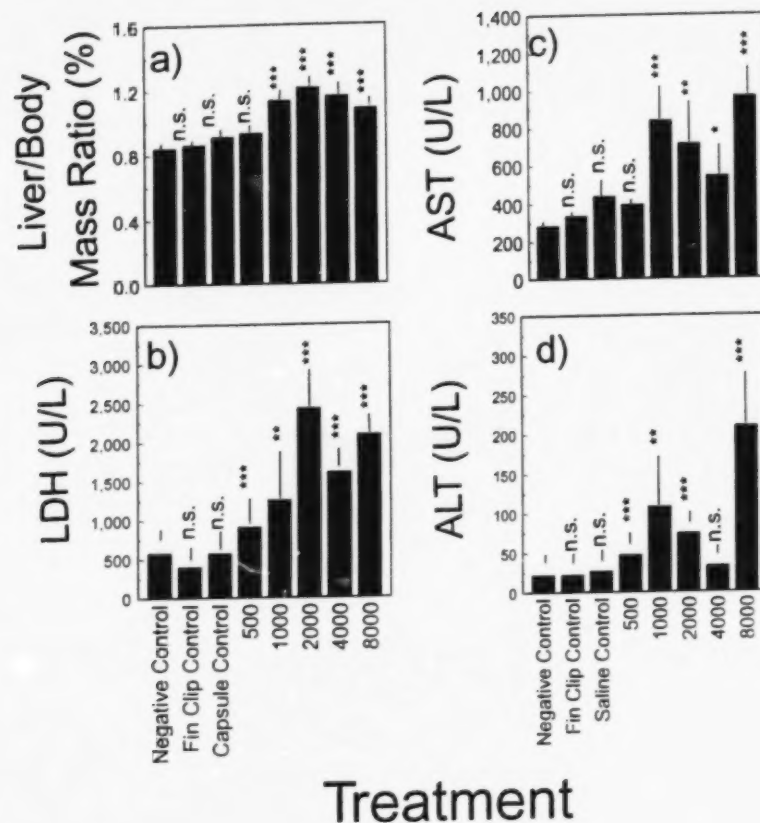


Figure 2. Average a) percent ratio of liver to body mass, and plasma activities (U L⁻¹) of b) lactate dehydrogenase, c) aspartic acid aminotransferase, and d) alanine aminotransferase in rainbow trout exposed orally to microcystin-LR. Fish in the negative control group were not manipulated, fin clip control fish were marked 72 h prior to the experiment, and capsule control fish received a gelatin capsule containing physiological saline. Microcystin-LR doses are reported as µg kg⁻¹. Vertical bars are standard error of the treatment mean. Levels of significance: n.s. = not significant ($P > 0.05$), * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.005$. Fin clip and capsule control groups were compared to the negative control treatment by one-way ANOVA. Each microcystin-LR treatment was compared to all controls combined, by *t*-test.

Figure 3. Light micrographs of livers from a) capsule control fish showing well-defined chords of hepatocytes with sinusoidal spaces, b) fish in the 500 $\mu\text{g kg}^{-1}$ microcystin-LR treatment showing swollen hepatocytes and obliteration of sinusoids, c) fish in the 2,000 $\mu\text{g kg}^{-1}$ treatment showing a focus of coagulative necrosis with hepatocytes containing pyknotic nuclei, and d) fish of the 8,000 $\mu\text{g kg}^{-1}$ treatment showing a large focus of degenerative hepatocytes typifying liquifactive necrosis. Bars equal 100 μm . All fish were from the oral exposure study.

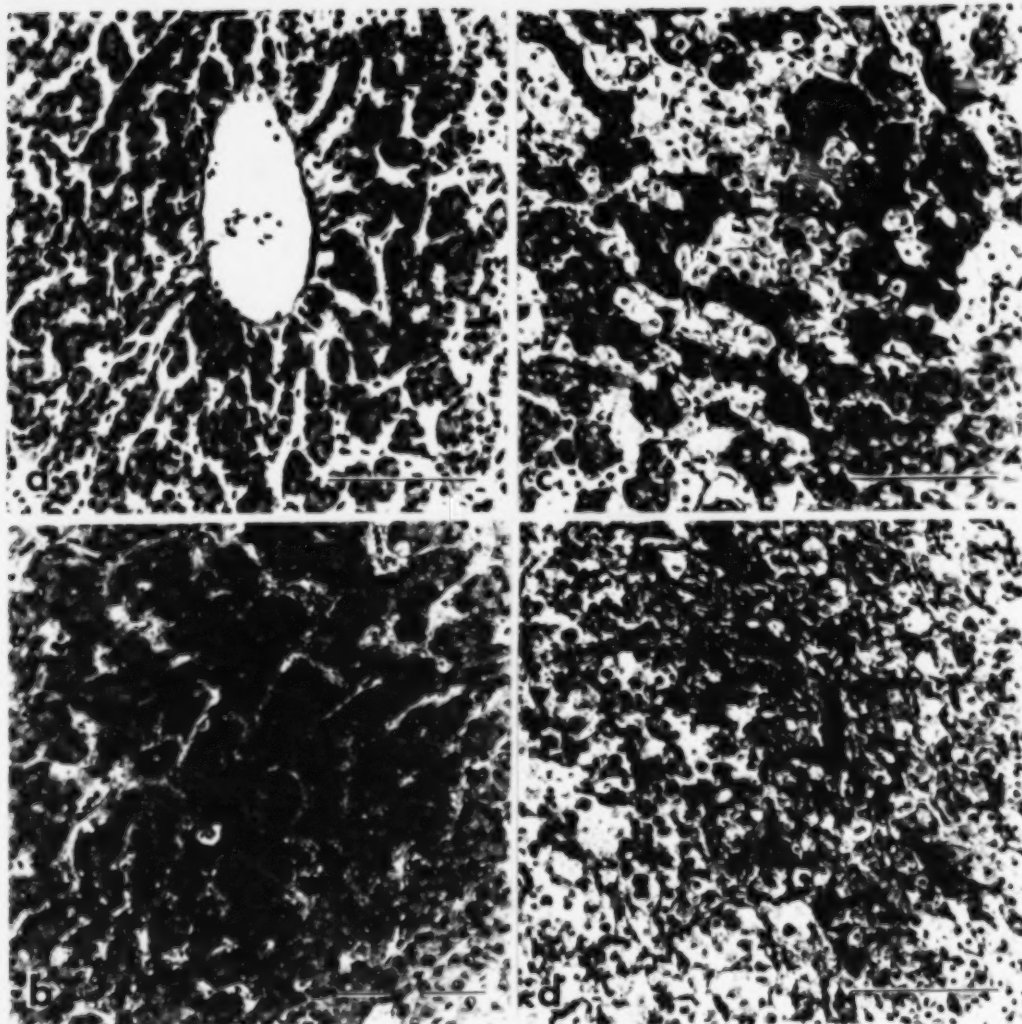


Figure 4. Electron micrographs of livers from a) capsule control fish showing irregularly-shaped and well-defined plasma membranes, and b) fish in the $8,000 \mu\text{g kg}^{-1}$ treatment showing a focus of liquifactive necrosis containing degenerative hepatocytes with extremely vacuolated cytoplasm and lysis of some plasma membranes. Bars equal $10 \mu\text{m}$. All fish were from the oral exposure trial.

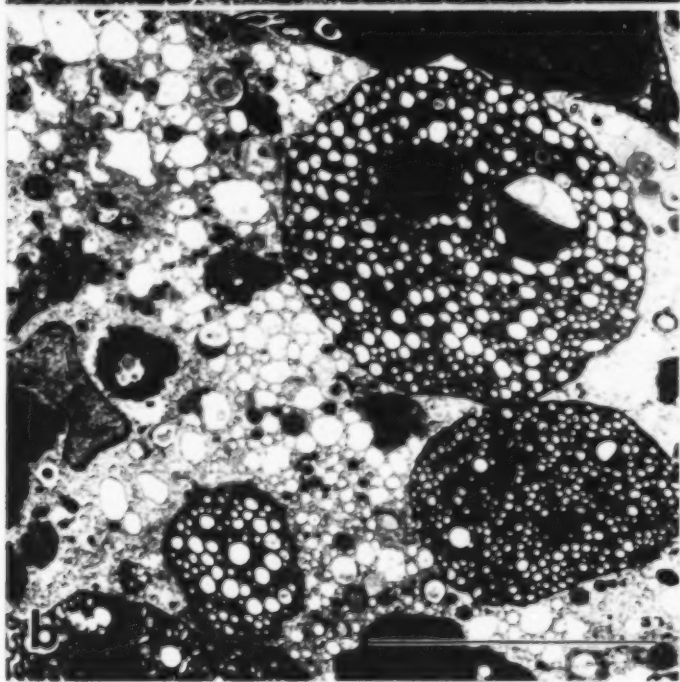
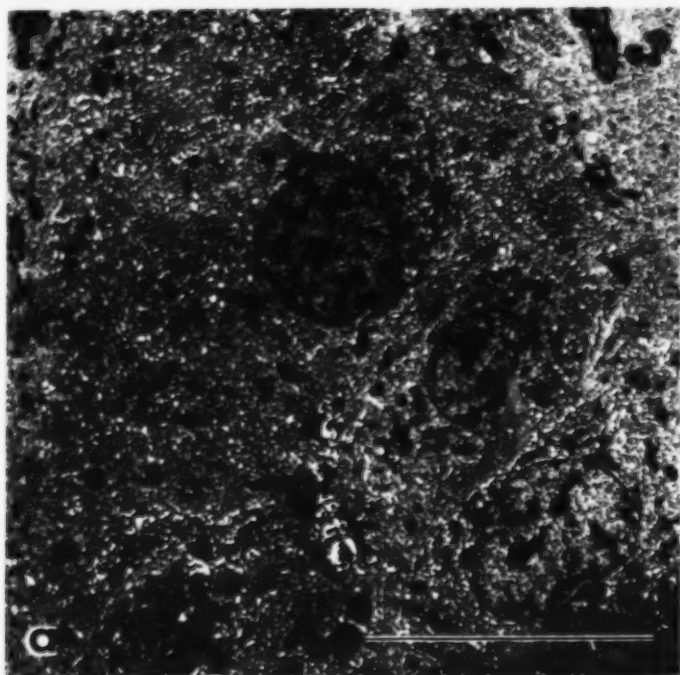


Figure 5. Light micrographs of the kidney from a) capsule control fish showing glomerulus fitting into Bowman's capsule snugly, and b) fish in the $8,000 \mu\text{g kg}^{-1}$ treatment showing dilation of Bowman's space. Bars equal $100 \mu\text{m}$. Fish were from the oral exposure trial.

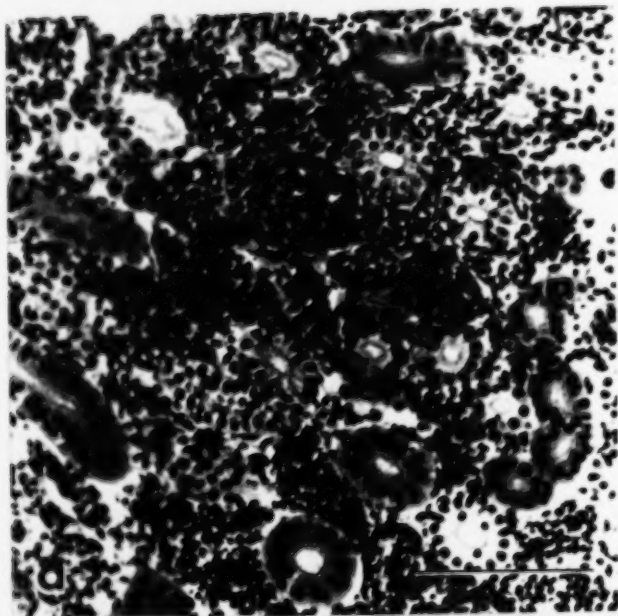


Figure 6. Electron micrographs of a kidney tubule from a) capsule control fish, and b) fish in the $8,000 \mu\text{g kg}^{-1}$ treatment showing mild coagulative necrosis of a tubule epithelium. Inset micrograph in b) shows a dilation of endoplasmic reticulum of a tubule epithelial cell causing vacuolization of the cell. Bars equal $10 \mu\text{m}$, except inset ($1 \mu\text{m}$). Fish were from the oral exposure trial.

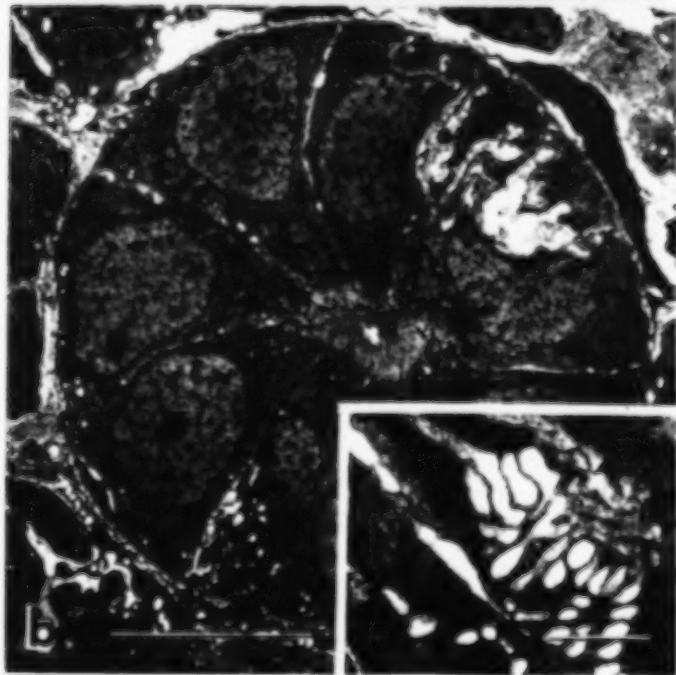
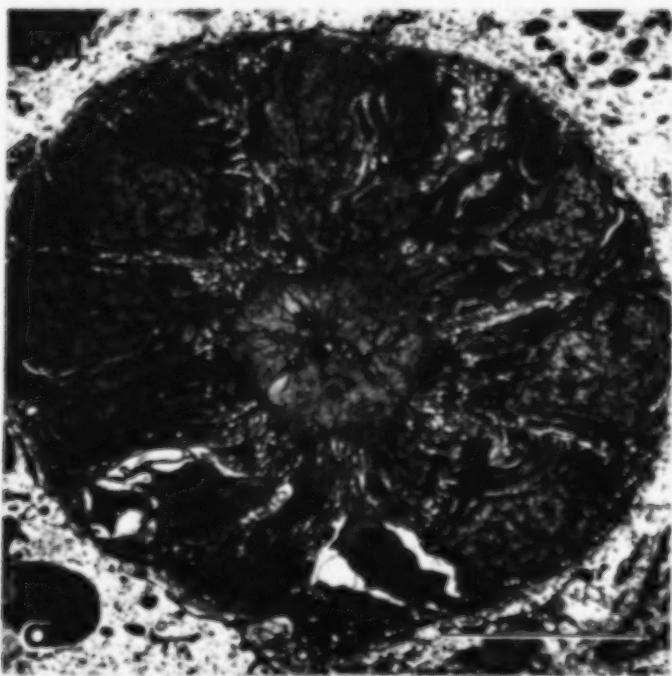




Figure 7. Light micrograph of pyloric caecum showing massive necrosis of the epithelium of the villi. Bar equals 100 μm . Fish was from the 500 $\mu\text{g kg}^{-1}$ treatment in the oral exposure trial.



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Chapter 9

General Discussion and Conclusions

Certain species of cyanobacteria produce potent neuro- and hepatotoxic compounds which, after accidental ingestion, can cause poisonings in animals (Schwimmer and Schwimmer 1968; Beasley et al. 1983; Pybus et al. 1986) and have been related to incidences of gastro- and hepatoenteritis (Lawton and Codd 1991) and primary liver cancer in humans (Carmichael 1994). These toxins appear to occur globally (reviewed by Carmichael et al. 1985). Although the cyanobacterial neurotoxin and hepatotoxin, anatoxin-a and microcystin-RR (MC-RR), respectively, were not detected in phytoplankton collected from water bodies in Alberta during an initial survey in 1990, the hepatotoxin MC-LR was detected by high performance liquid chromatography (HPLC) at least once, and in some cases almost continually in 24 of 28 lakes, six of 11 farm dugouts and one of three storm water retention ponds surveyed for up to 5 yr (1990 to 1994). Therefore, the occurrence of MC-LR in central Alberta water bodies was widespread. The identity of MC-LR in phytoplankton was also confirmed by fast atom bombardment-mass spectrometry (FAB-MS) on a composite phytoplankton sample collected from Coal Lake in 1990.

MC-LR concentration in the phytoplankton, expressed as either $\mu\text{g g}^{-1}$ plankton biomass or as $\text{ng cellular toxin L}^{-1}$ of lake water, from three intensively studied lakes (Coal, Driedmeat and Little Beaver), was highly variable both spatially and temporally. This is the first study to examine toxin variability in lakes in detail by sensitive methods of detection (HPLC). The variability in concentration was most strongly correlated to changes in the abundance and biomass of the cyanobacterium, *Microcystis aeruginosa* (r up to 0.72), although toxin concentration within the *M. aeruginosa* cells was also variable. The presence of *M. aeruginosa* in phytoplankton was a relatively consistent indicator that MC-LR would be detected. Correlation analyses were performed on data collected from up to 12 eutrophic lakes and for up to five yr to examine the limnological factors that may influence the *in situ* concentration of MC-LR in phytoplankton. MC-LR (expressed as $\mu\text{g g}^{-1}$ *M. aeruginosa*) and *M. aeruginosa* biomass was strongly correlated with total

phosphorus concentration. However, *M. aeruginosa* biomass alone was related by a negative exponential relationship to the ratio of total nitrogen to total phosphorus (TN:TP) and forms of inorganic nitrogen ($\text{NO}_3^- + \text{NO}_2^-$, NH_4^+). Therefore, some factors appeared to influence MC-LR in phytoplankton by enhancing both *M. aeruginosa* biomass and toxin concentration in the cells while others influenced one component only. The production of MC-LR in phytoplankton therefore appeared to be regulated primarily by nitrogen and phosphorus. These data suggest that changes in nutrient loading to lakes or a change in the TN:TP ratio, possibly as a result of land use practices (e.g. agriculture, forestry, urbanization) may enhance MC-LR concentrations in lakes.

Only two studies have examined whether microcystins can accumulate in organisms associated with aquatic food webs. A microcystin analogue was detected in mussels (*Anodonta cygnea*) after exposure to a hepatotoxic culture of *Oscillatoria agardhii* in a laboratory experiment (Eriksson et al. 1989) and in zooplankton collected from Lake Kasumiguara, Japan (Watanabe et al. 1992). Four lakes in Alberta, varying in trophic status from oligo-mesotrophic to hypereutrophic, were surveyed for up to three yr to examine MC-LR concentrations (by HPLC and protein phosphatase bioassay) in some compartments of the aquatic food webs (water, phytoplankton, zooplankton and macroinvertebrates, and to a limited extent, fish). MC-LR concentration increased with lake trophic status, tracking the abundance of *M. aeruginosa*. MC-LR concentration in zooplankton (up to $67 \mu\text{g g}^{-1}$ of biomass) was correlated to toxin concentration in the phytoplankton ($r=0.69$). Of nine groups of macroinvertebrates assessed, MC-LR was detected only in gastropods (at concentrations up to $120 \mu\text{g g}^{-1}$). These data suggest that invertebrates which feed on phytoplankton containing MC-LR can also accumulate significant concentrations of the toxin. A lack of detectable MC-LR in the livers of northern pike and white suckers from Driedmeat Lake likely indicated that most of the toxin was bound to protein phosphatases in the liver. I believe that MC-LR present in invertebrates (zooplankton, gastropods) could be transferred to other organisms (fish, birds), although the pathological effects in these, and other organisms, are not known.

The pathological effects of MC-LR in mammals, however, have received a considerable amount of attention. This is likely a result of the need to understand the

toxins' impact on economically-important animals such as cattle, sheep, and pigs, which can be highly susceptible to the toxin (Carmichael et al. 1977; Jackson et al. 1984; Beasley et al. 1986). Very few studies have examined the toxicity and pathological effects of MC-LR in fish (Phillips et al. 1985; Raberg et al. 1991; Andersen et al. 1993), and only one by a natural route of exposure (e.g., oral exposure, Tencalla et al. 1994).

I examined the intraperitoneal toxicity and pathological effects of MC-LR in rainbow trout and in mice. Both intraperitoneal exposure in mice and intraperitoneal and oral exposure to MC-LR in rainbow trout caused dose-dependent mortality, but fish appeared to be less susceptible (e.g., some fish survived intraperitoneal doses up to 800 $\mu\text{g kg}^{-1}$, 16 times higher than the reported LD_{50} in mice) and survived a lethal dose much longer than mice. The main pathological effects were observed primarily in the liver in both species, but the characteristic or hallmark lesion differed. The prominent liver lesion in mice was massive hemorrhage while lesions in fish consisted of coagulative or liquifactive hepatocyte necrosis, whereby the plasma membranes of hepatocytes ruptured, leaving a milieu of cellular debris. Increased liver weight (relative to the body mass) occurred in both species and was caused by pooling of blood in the liver (i.e., liver hemorrhage) in mice but was probably caused by water retention in the fish. Hemorrhage was rarely observed in fish livers. Elevated plasma activities of lactate dehydrogenase, aspartic acid aminotransferase and alanine aminotransferase in fish after intraperitoneal or oral exposure to MC-LR were indicative of the pathological damage observed in the liver at the histological (light microscopy) and ultrastructural level of resolution. Liver damage in fish caused by oral exposure to MC-LR was less severe than approximately equivalent doses by intraperitoneal exposure, suggesting that the uptake of MC-LR from the gastrointestinal tract was not 100% efficient. Therefore, exposure to MC-LR caused significantly different pathological effects in the liver of fish and mice. The reasons for this difference are unknown.

In addition to liver damage, intraperitoneal and oral exposure to MC-LR in fish caused kidney damage consisting of coagulative and liquifactive necrosis of tubular epithelium and a dilation of Bowman's space. Kidney damage was not observed in mice exposed to MC-LR. Oral exposure to MC-LR also caused severe necrosis of the villi of

pyloric caecae and the intestine in fish. My studies on fish indicate that MC-LR is highly toxic to rainbow trout and that oral exposure of fish to MC-LR, possibly through feeding on organisms (zooplankton, gastropods) that have accumulated the toxin by their own feeding activities, could cause adverse health effects in fish including damage to the liver, kidney and gastrointestinal tract.

In summary, MC-LR was commonly found in eutrophic and hypereutrophic lakes in central Alberta and toxin concentration in phytoplankton appeared to be influenced most greatly by the nutrients nitrogen and phosphorus. Based on concentrations of MC-LR detected in aquatic invertebrates and pathological effects of the toxin by oral exposure in rainbow trout, chronic exposure of fish species (e.g., lake whitefish, northern pike, white sucker, yellow perch) from feeding on toxin-contaminated invertebrates could lead to pathological lesions in the liver, kidney and gastrointestinal tract of fish. This may explain the slow growth rates of some fish species in some hypereutrophic lakes which regularly contained MC-LR (e.g., Driedmeat Lake).

This thesis has identified several areas of study which require further attention. These include: 1) an examination of the relationships between environmental variables and cyanobacterial toxin concentration in lakes over a wide geographic area to evaluate the generality of the relationships observed in my studies, which are from a semi- and arid region where MC-LR is the dominant cyanobacterial toxin, 2) studies which determine the effect of the ratio of nitrogen to phosphorus on cyanobacterial toxin production at laboratory, mesocosm (e.g., limnocorral) and ecosystem-level scales, 3) an evaluation of the effects of land disturbance (e.g., agriculture, forestry, fire) in lake watersheds on MC-LR production, and other cyanobacterial toxins, in lakes, 4) an examination of the uptake, depuration and pathological effects of MC-LR and other cyanobacterial toxins in aquatic invertebrates such as various zooplankton species and gastropods, and 5) studies to determine the basis for the significantly different pathological effects of MC-LR in mammals and fish.

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